The non-apoptotic function of Caspase-8 in endothelial precursor cells

Dissertation

zur Erlangung des Doktorgrades

der Naturwissenschaften

vorgelegt beim Fachbereich Biowissenschaften

der Johann Wolfgang Goethe-Universität

in Frankfurt am Main

Dörte Scharner

born in Stralsund/ Mecklenburg-Vorpommern

Frankfurt, 2009

GOETHE UNIVERSITÄT FRANKFURT AM MAIN

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Dekan: Herr Professor Dr. Volker Müller

1.Gutachter: Frau Professorin Dr. Anna Starzinski-Powitz

2.Gutachter: Frau Professorin Dr. Stefanie Dimmeler

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Contents

1. Introduction

The vascular system is a network of vessels that carries blood and lymph through the body. The system can be divided into different parts: heart, macro-circulation, micro-circulation and lymph vascular system. The vessels of the blood circulatory system are divided into arteries, veins and capillaries. The artery blood vessels carry oxygen-saturated blood and nutrients from the heart to the body. The venous system carries deoxygenated blood from the body back to the heart and lung to be released as carbon dioxide. The small capillaries between arteries and veins provide nutrients, immune cells and oxygen to every cell in the surrounding tissue as well as transport waste products and pick up carbon dioxide to secrete it from the body. The essential oxygen and carbon dioxide are exchanged through the respiratory system.

1.1 Vasculogenesis, Angiogenesis and Arteriogenesis

Arteries, veins and capillaries form a complex network of branched structures in adults. The development of this network is mediated by three different processes: vasculogenesis, angiogenesis, and arteriogenesis.

1.1.1 Vasculogenesis

The early stage of blood vessel formation is called vasculogenesis. During embryonic development, undifferentiated precursor cells, so-called hemangioblasts form aggregates and differentiate into hematopoietic precursors and endothelial cells (ECs) to form a primitive vascular network of small capillaries, the vascular plexus (Choi, Kennedy et al. 1998), reviewed in (Carmeliet 2000). Vascular endothelial growth factor (VEGF, also known as KDR or Flk1) and basic fibroblast growth factor (bFGF) stimulate the differentiation of endothelial cells into arterial and venous ECs. This fate of endothelial precursor cells to become integrated into arteries or veins is further supported by gene members of the Notch family, the Hox (Homeobox) family and members of GATA and ephrin family (Gale and Yancopoulos 1999). For

instance, mutations of Notch ligand delta-like (DLL)4 leads to a defective arterial development and upregulation of venous markers (Duarte, Hirashima et al. 2004) (Krebs, Shutter et al. 2004). Vessel growth, however, is not only restricted to the embryo. Endothelial precursors contribute to blood vessel growth also in ischemic, inflammed and malignant tissue in the adult. For example, endothelial precursor cells, mesoangioblasts and multipotent adult precursors in the bone marrow and peripheral blood are stimulated for mobilization and differentiation by VEGF, placental growth factor (PLGF), angiopoietin (Ang)-1 and cytokines to support vasculogenesis of new blood vessels (reviewed (Carmeliet 2003)).

1.1.2 Angiogenesis

Remodelling, sprouting and branching from pre-existing vessels to new capillaries is defined as angiogenesis. Different mechanisms like activation, migration and proliferation of endothelial cells and endothelial progenitor cells are involved in this angiogenic process forming a higher organized and stereotyped vascular network. Signalling through the hypoxia-inducible transcription factor (HIF) induces vasodilation of the vessels and upregulates angiogenic genes like VEGF, angiopoietin-2, nitric oxide synthase, TGFβ-1, interleukin-8 and Tie1 which are crucial for cardiovascular homeostasis by supporting endothelial cell proliferation, migration and expression of adhesion molecules. (Carmeliet 2000) (Carmeliet 2003) (Aicher, Heeschen et al. 2003) (Carmeliet 2005). The partial knockout of the VEGF gene leads to a vascular defect resulting in embryonic lethality (Carmeliet, Ferreira et al. 1996) (Ferrara, Carver-Moore et al. 1996). Furthermore, ECs communicate within the vessel wall. Blood flow and increase in blood pressure lead to interactions between the cytoskeleton of ECs and the surrounding extracellular matrix (ECM). ECs start to migrate and adhere at different places. Sprouting and remodelling of vessels causes remodelling of ECM by proteinases like plasminogen activators, matrix metalloproteinases (MMPs) and cathepsins. Vessel stabilization is reached by recruiting mural cells (smooth muscle cells and pericytes) which are activated by shear stress responsive gene transcription, Tie-2 receptor, PDGF, TGF-β and PlGF

(Carmeliet 2003). Some processes of vascular embryonic development take place in neoangiogenesis in the adult or as neovascularization in injured or ischemic tissue (Carmeliet 2003). Insufficient or abnormal vessel growth can cause many diseases like hypertension, neurodegeneration and osteroporosis.

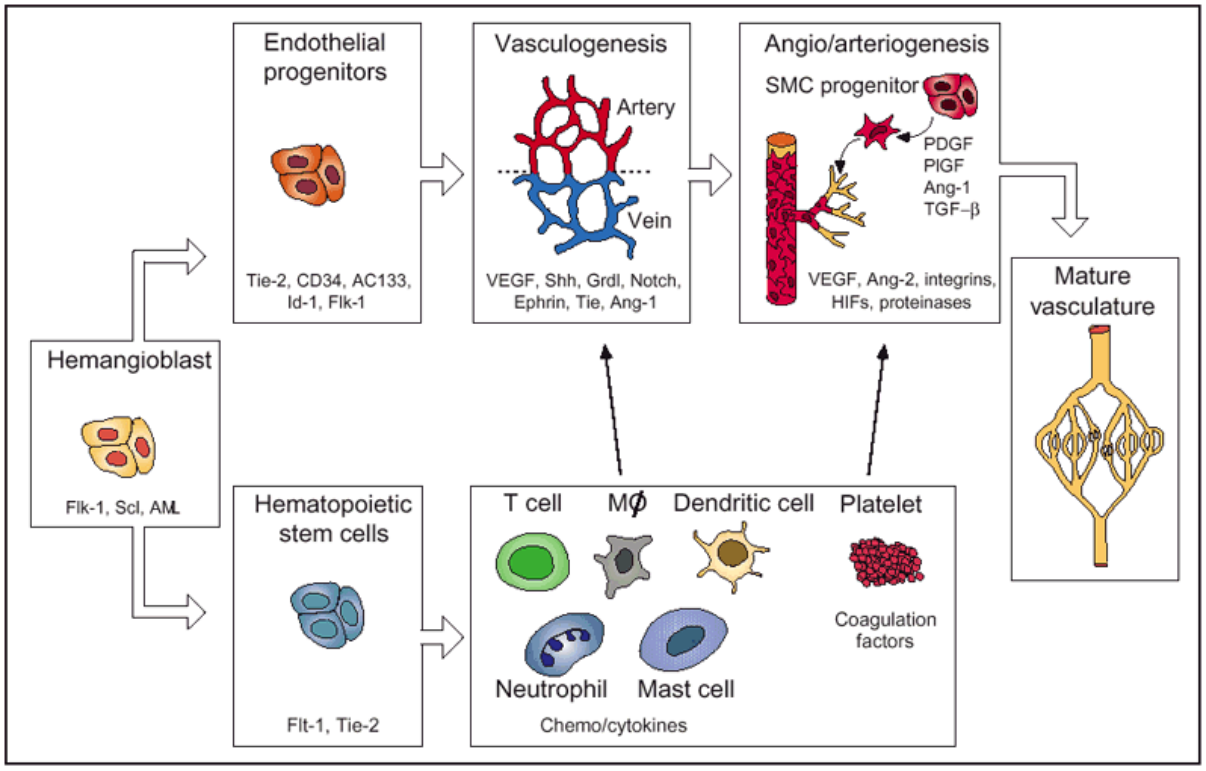


Figure 1.2.1: Vascular network formation by vasculogenesis, angiogenesis and arteriogenesis

Endothelial progenitor cells from a common hemangioblast form a primitive vascular capillary plexus after differentiation into arterial and venous ECs dependent on the stimuli. This early process is called vasculogenesis. Smooth muscle cells surround sprouting vessels for stabilization. Hematopoietic stem cells support angiogenesis by releasing angiogenic factors, differencing into various cell types and endothelial cells (Carmeliet 2003).

1.1.3 Arteriogenesis

Arteriogenesis is a consequence of physical forces such as longitudinal, circumferential and radial wall stress caused by increased blood pressure (Heil, Eitenmuller et al. 2006). Smooth muscle cell proliferation, differentiation and migration increases collateral growth and results in sprouting and migration alongside pre-existing vessels. Chemoattracted monocytes adhere within the collateral wall to support remodelling and proliferation of vascular wall cells. Eventually, they are also important for processes in arteriogenesis (Carmeliet 2003) (Heil, Eitenmuller et al. 2006).

1.2 Structure and maturation of blood vessels

As previously mentioned, during embryonic development the nascent vascular capillary network develops by vasculogenesis and angiogenesis. Nascent and mature vessels differ in capillary (small), artery and vein (big vessels) morphology. Vessel development starts with a channel of ECs (Fig.1.3.1a). These nascent capillaries consist of the EC tube surrounded by a thin layer of pericytes which are enveloped by an EC basement membrane (Fig.1.3.1b). The arterial-venous fate is influenced by the local metabolism (e.g. hypoxia, pH) and the mechanical environment (e.g. shear stress, hydrostatic pressure). Pericytes and vascular SMCs cover the basal capillary structure embedded into elastic lamina and basement membrane (Fig.1.3.1c). In order to tissue and organ specificity, the walls are divided into three layers. The inner part, the so-called intima is based on ECs. SMCs form the media, whereas the outer adventitia is composed of fibroblasts, extracellular matrix and elastic lamina (Fig.1.3.1d). Communication between ECs and as well as ECs and surrounding mural cells is mediated by gap junctions, cadherins and tight junctions, which are also important for the tissue-blood-barrier in brain and retina (Jain 2003).

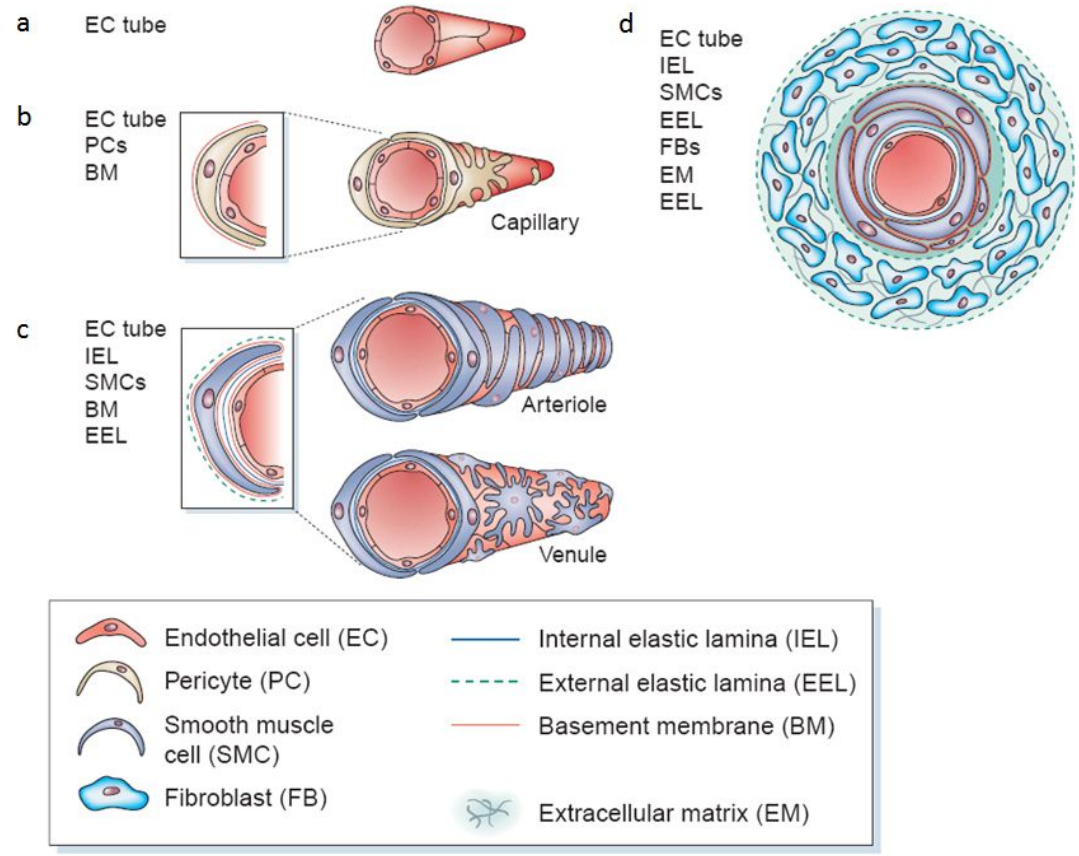


Figure 1.3.1: Vessel maturation during different stages in development

(a) New developing vessels consist of a monolayer of endothelial cells. At this point, they further expand into capillaries, arteries or veins. (b) Capillaries, based on the endothelial tube (a), are surrounded by a thin layer of pericytes embedded into a basement membrane. This kind of vessel structure is involved in import and export between blood and tissue (c) Developing arterioles and venules are covered with vascular smooth muscle cells carrying their own basement membrane and are embedded into an external elastic lamina. (d) Larger vessels are made of three layers: intima, media and adventitia. Modified after (Jain 2003)

1.3 Endothelial precursor (progenitor) cells

The existence of so-called endothelial precursor (progenitor) cells was first described by Asahara and co-workers. They isolated putative $CD34^+$ endothelial progenitor cells from human peripheral blood using magnetic microbeads (Asahara, Murohara et al. 1997). After ex vivo cultivation on fibronectin matrix, this heterogeneous cell

population differentiates into cells with endothelial character expressing endothelial markers like CD133, CD34 and the VEGFR-2 (Peichev, Naiyer et al. 2000). In general, cells which express CD133, a hematopoietic stem cell marker, are mainly localised in the bone marrow (Yin, Miraglia et al. 1997) and show no expression of vascular endothelial cadherin and von Willebrand factor. Furthermore, they found more mature and differentiated EPCs in the pheripheral circulation of adults but missing CD133 surface expression. It is conseivable that these cells have their origin in the bone marrow (Shi, Rafii et al. 1998) loosing the marker CD133 probably during transmigration into the blood circulation. These circulating EPCs lacking CD133, express CD34, VEGFR-2, CD31, VE-cadherin, von Willebrand factor and incorporate Dil-Ac-LDL. These findings supported the assumption that EPCs are bone marrow-derived with a hemangioblast origin (Loges, Fehse et al. 2004), migrate into the circulating blood system and differentiate further to cells with endothelial character. But EPC were also isolated from fetal liver or umbilical cord blood (Yin, Miraglia et al. 1997) (Murohara, Ikeda et al. 2000). The number of $CD133^+$ cells in the blood can be increased by mobilizing cells from the bone marrow by injection of granulocyte colony stimulating factor (G-CSF) (Peichev, Naiyer et al. 2000). In addition, it was shown that these $CD133^+$ cell populations are able to differentiate into a hematopoietic lineage and into endothelial cells in the presence of the cytokines VEGF and stem cell growth factor (SCGF) (Gehling, Ergun et al. 2000). Because the definition of EPCs has a wide range and differs between different investigator groups, there have been efforts to define the heterogeneity in more detail and to distinguish between early and late EPCs (Gulati, Jevremovic et al. 2003) (Hur, Yoon et al. 2004). Hur et. al isolated total mononuclear cells from human blood and gained early spindle shaped EPCs with a lifespan of 4 weeks as well as late outgrowth EPCs with a paving stone structure and a life expectancy up to 12 weeks. Furthermore, these two populations differ in secretion of angiogenic cytokines, surface marker expression and in *ex vivo* assay outcomes but show similarity in their *in vivo* vasculogenic capacity. However, so far there is no clear definition for EPCs from peripheral blood. In summary, endothelial precursor cells are a mixture of cells with a high variety in

differentiation, strongly dependent on the isolation protocol as well as *ex vivo* cell culture conditions (Seeger, Tonn et al. 2007).

1.4 Endothelial precursor (progenitor) cells for therapeutic use

During the last decade, experimental investigations have demonstrated that EPCs improve neovascularisation (Rafii and Lyden 2003). Cell therapy is a promising option to enhance endothelial repair and tissue perfusion after ischemia. Transplantation of human EPCs into nude mice enhanced the blood flow recovery and capillary density within the hind limb ischemic area (Kalka, Masuda et al. 2000) as well as myocardial neovascularization and improved left ventricular function after myocardial infarction (Kawamoto, Gwon et al. 2001). Additional mouse experiments have proven that EPCs in the blood are mobilized from the bone-marrow by ischemic events or are attracted by cytokines like GM-CSF (granulocyte macrophage- colony stimulating factor). Accordingly, mobilized cells are attracted to the injured myocardium after acute myocardial infarction (Takahashi, Kalka et al. 1999) (Orlic, Kajstura et al. 2001). The inhibition of specific proteases like cathepsin L plays a critical role for the invasive and functional capacity of EPC-mediated neovascularisation in ischemic tissue (Urbich, Heeschen et al. 2005). In patients with acute myocardial infarction, intracoronary infusion of autologous adult progenitor cells either from the bone-marrow or from the blood resulted in significant increase of LV (left ventricular) ejection fraction, normalization of flow reserve and coronary wall motion as well as in reduction of the end systolic LV volume within four month (Assmus, Schachinger et al. 2002). Based on this study, intracoronary administration of progenitor cells derived from the bone marrow, combined with optimal reperfusion by stent therapy and medical treatment, improved the recovery of global and regional left ventricular contractile recovery after myocardial infarction in a multicenter trail (Schachinger, Erbs et al. 2006). Different drugs like AMD-3100 (De Clercq 2005), statins (Vasa, Fichtlscherer et al. 2001), EPO (Heeschen, Aicher et al. 2003) and estrogens (Strehlow, Werner et al. 2003) interfere with the number of circulating EPCs, migration and adhesion capacity, as well as EPC mobilization and homing.

Besides pharmacological agents and molecules, physical exercise influences EPC mobilization supports vascular repair (Sandri, Adams et al. 2005).

1.5 EPC function in diseases

Endothelial precursor cells are correlated with some cardiovascular diseases. Increased cardiovascular risk factors in patients result in decreasing numbers of circulating progenitor cells by influencing their mobilization from the bone-marrow and enhancing EPC senescence (Hill, Zalos et al. 2003). In patients with diabetes mellitus, EPCs have impaired functions for neovascularization, e.g. in cell to cell and cell to matrix adhesion, proliferation and tubulization with endothelial cells (Tepper, Galiano et al. 2002). Kissel et al. showed in their studies that patients with coronary artery disease (CAD) are less able to mobilize progenitor cells into the blood, which correlates with functional limitation of hematopoietic progenitor cells in the bone marrow niche and impairment of MMP-9 (matrix metalloproteinase-9) (Kissel, Lehmann et al. 2007). On the other hand, bone-marrow derived VEGF-responsive cells like EPCs play a crucial role in tumor angiogenesis by supporting neovascularization (Lyden, Hattori et al. 2001). Tumors as a tissue with own growth and independent development release growth factors like VEGF and chemokines like CCR2 and CCR5 (a subfamily of chemokine receptors) from neovessels for chemokine-dependent attraction of EPCs (Spring, Schuler et al. 2005). These new strategies for healing, restoration and prevention in cardiovascular diseases could be useful to find new therapeutic approaches (Kawamoto and Losordo 2008).

1.6 Caspases and their function

Apoptosis is an evolutionary conserved programmed cell death which is highly important in embryonic development, tissue formation and the elimination of harmful cells (Lamkanfi, Declercq et al. 2002). This form of controlled death is dependent on the activity of caspases, a family of pro-apoptotic enzymes (Thornberry 1998). These

cysteinyl aspartate proteinases cleave their substrates after a specific aspartate residue (Thornberry and Lazebnik 1998). Dependent on the species different types of caspases have been described so far. In mammalian, 14 caspases have been identified (11 in human) and ten in mouse, seven in *Drosophila melanogaster* and four in *Caenorhabditis elegans (Lamkanfi, Declercq et al. 2002)*. The cell synthesizes caspases as precursors called zymogens without or low catalytic activity. Caspases are divided into two classes: initiators and effectors. They contain an N-terminal prodomain like the caspase recruiting domain (CARD) or the death effector domain (DED) (Fig.1.6.1.).

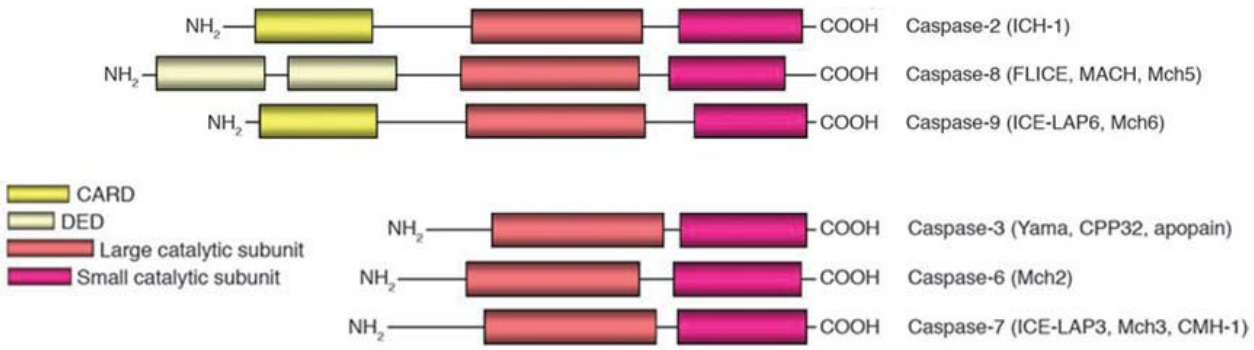


Figure 1.6.1: Schematic illustration of the two caspase classes

Inactive proenzymes are composed of an amino-terminal prodomain, a large subunit, a small subunit and linker regions between them with an aspartate residue as internal cleavage sites for catalytic activation. Modified after (Lavrik, Golks et al. 2005)

Initiator caspases like caspase-2, -8 and -9 are autoactivated under apoptotic conditions by linking the prodomain of the caspase to death signalling complexes, also called “proximity-induced” activation. As a complex, caspases undergo dimerization to form an active conformation as a heterotetramer containing two large and two small subunits of two procaspases. Investigated multimeric caspase complexes are the apoptosome (caspase-9 activation) (Riedl and Salvesen 2007), the inflammasome (caspase-1 activation), the piddosome (activates caspase-2) (Tinel and Tschopp 2004) and the death-inducing signalling complex (called DISC,

which activates caspase-8) (Kischkel, Hellbardt et al. 1995) (Peter and Krammer 2003). They are further classified into the extrinsic pathway or intrinsic activation (Fig.1.6.2.).

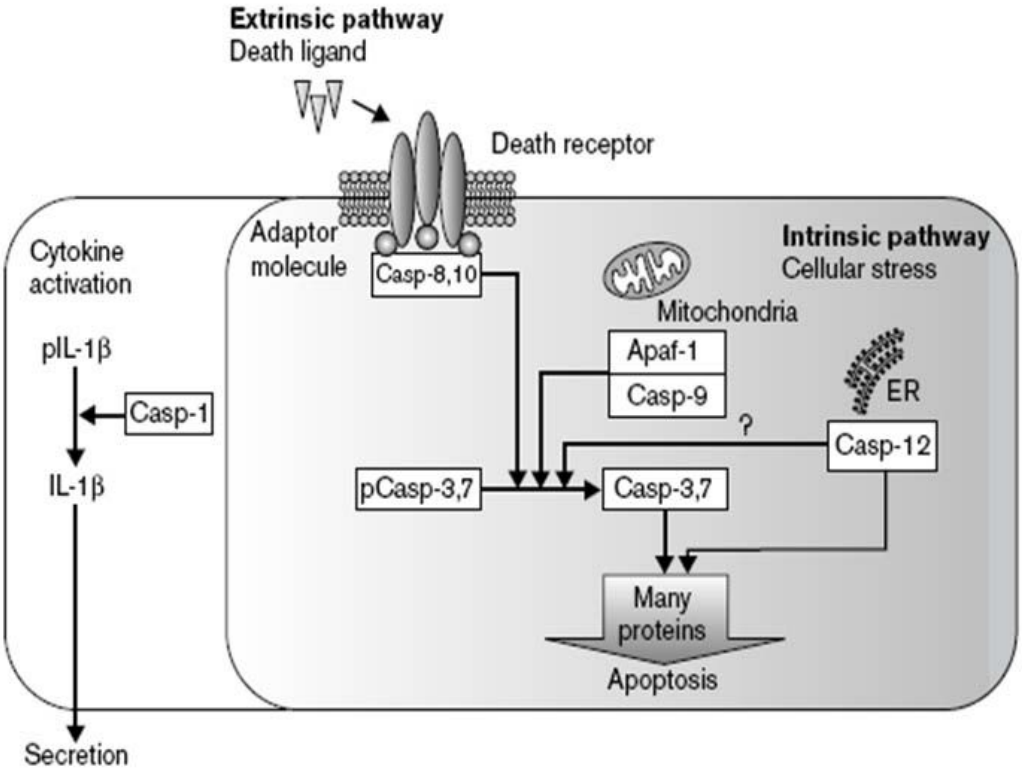


Figure 1.6.2: Schematic illustration of the main extrinsic and intrinsic apoptosis induction pathways

Two alternative pathways can activate the apoptotic induction. The extrinsic pathway is controlled by death receptors (e.g. Fas) on the cell surface and is binding of specific death ligands like FasL and the intrinsic pathway by mitochondria. Both pathways lead to activation of an initiator caspase: caspase-8 for the extrinsic pathway and caspase-9 at the apoptosome for the intrinsic pathway. Then, the initiators activate downstream (executioner) caspases resulting in cleavage of the death substrates. A positive feedback couples these two pathways. For example, cleavage of procaspase-3 by caspase-9 activates in a feedback loop caspase-8 and even cleavage of more caspase-3. This entails an amplification of the apoptotic signal. Illustration by (Grutter 2000)

Key effector caspases like caspase-3, -6 and -7 are missing the prodomain. Consequentially, they are not capable of autoactivation and are dependent on the

cleavage by active initiator cysteine proteases like caspase-2, -8 or -9 after a specific internal aspartate residue. Initiator caspases in *Drosophila melanogaster* are DREDD with a DED-prodomain like caspase-8 (mammals) and DRONC with a long amino-terminal CARD domain similar to caspase-2 or -9. DRICE, DCP-1 and DECAY are highly homologue to each other and are close relatives of the mammalian effector caspase-3 (Kumar and Doumanis 2000). Onces activated, effector caspases cleave a wide variety of intracellular targets resulting in cell death. Some caspase substrates in the apoptotic way are ROCK1 (Sebbagh, Renvoize et al. 2001), Rac GTPases (Zhang, Zhang et al. 2003), myosin light chain kinase (MLCK) (Petrache, Birukov et al. 2003) and plectin (Stegh, Herrmann et al. 2000) which are involved in cytoskeleton formation.

1.7 Non-apoptotic functions of caspases

In the last years, several publications about the non-apoptotic functions of caspases have been published. Loss-of-function in *Drosophila* DREDD, a caspase related to caspase-8, prevents bacterial infection (Leulier, Rodriguez et al. 2000). In *Drosophila* spermatozoid differentiation, caspase-3-like effector caspase DRICE is important for elimination of the cytoplasm regulated by a specific cytochrome c resulting in functional sperm development (Arama, Agapite et al. 2003). Furthermore, border cell migration in Drosophila ovary was supported by Drosophila inhibitor of apoptosis 1 (DIAP1) whereas loss of the according gene caused inhibition of migration in this Rac-dependent cell process (Geisbrecht and Montell 2004). Recently, caspase-11 was shown to trigger actin depolymerisation during inflammatory events (Li, Brieher et al. 2007). Additionally, the executioner caspase-3 targets Nanog, a transcriptional regulator, by cleavage and thus mediates embryonic stem cell differentiation (Fujita, Crane et al. 2008) as well as influencing hematopoietic stem cell homeostasis via cytokine-influenced signalling (Janzen, Fleming et al. 2008). Furthermore, executioner caspases are necessary for lens development (caspase-3) (Zandy, Lakhani et al. 2005), erythroblast (Zermati, Garrido et al. 2001), platelet (caspase-3

and -9) (De Botton, Sabri et al. 2002) and embryonic keratinocyte differentiation (caspase-3) (Okuyama, Nguyen et al. 2004).

1.8 Caspase-8

Intracellular caspase-8 activation in the cell death-inducing pathway by the TNF (tumor necrosis factor) receptor family triggers apoptosis (Boldin, Goncharov et al. 1996). Investigations in the last years revealed non-apoptotic functions of caspase-8. This cysteine protease is involved in embryonic development, monocyte differentiation, T cell activation as well as T and B cell proliferation. Wallach and co-workers investigated the importance of caspase-8 for apoptosis by generating caspase-8 deficient mice (Varfolomeev, Schuchmann et al. 1998). Homozygous deletion of the caspase-8 genes leads to embryonic death around day E11.5 with an abnormal phenotype. Caspase-8 deficiency resulted in increased erythrocytosis in the liver, hyperaemia in many organs, e.g. in the brain and in the lens of the eye, and blood vessels. Furthermore, histological investigation showed defects in the heart muscle and neural tube development as well as a reduction in the hematopoietic precursor pool. *Ex vivo* cultured fibroblasts of caspase-8 deficient embryos were resistant to death induction by TNF receptors, Fas/Apo1 and DR3 compared to heterozygous littermates (Varfolomeev, Schuchmann et al. 1998). Caspase-8-deficiency constricted to the T-cell lineage in mice revealed functional importance of this cysteinyl protease in T-cell homeostasis, immune response and resulted in infiltration of T cells into organs like liver, lung and kidney in older mice (Salmena, Lemmers et al. 2003) (Salmena and Hakem 2005). Humans with inherited genetic deficiency in caspase-8 show defects in T, B and NK (natural killer) cell activation and autoimmunity (Chun, Zheng et al. 2002). Additionally, the specific transcription factor NF-κB (Nuclear Factor κB) activation is dependent on enzymatic activity of caspase-8 induced by bacterial and viral antigens like Fc receptors or Toll-like receptor 4 but is independent of TNF (Tumor necrosis factor) (Su, Bidere et al. 2005). Furthermore, fetal liver hematopoietic stem cells are not able to proliferate without caspase-8 enzymatic activity (Pellegrini, Bath et al. 2005). Cell-restricted deletion of caspase-8 revealed embryonic lethality only in full knockout mice and in mice with a

specific depletion of Caspase-8 in endothelial cells (Varfolomeev, Schuchmann et al. 1998) (Kang, Ben-Moshe et al. 2004). Deletion of caspase-8 in a tissue- and time-specific manner by different promoters like Mx1, LysM, Lck and Alb leads to functional deviation in the process of various cell types like hematopoietic precursor, monocytes, T and B cells and liver regeneration. Caspase-8 knockout mice as well as depletion by the endothelium-specific Tie1 promoter revealed strong evidence for a non-apoptotic role during embryogenesis evidenced by failed yolk sac vasculature (Kang, Ben-Moshe et al. 2004). In the context of therapeutical approach, it is specifically interesting that fibroblast from caspase-8 deficient mice show decreased cell motility and calpain functionality resulting in downregulation of Rac activity and lamellipodial organisation in tumor growth (Helfer, Boswell et al. 2006).

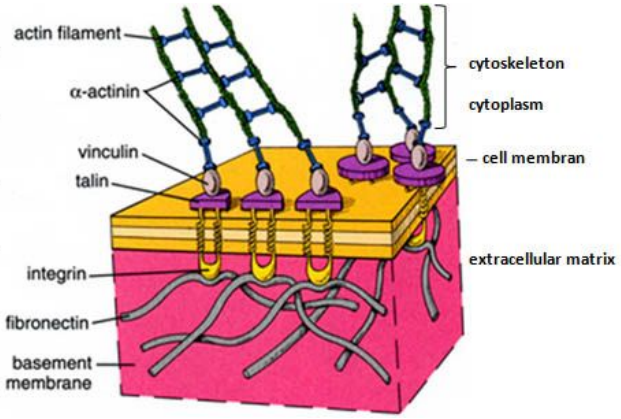
1.9 Integrins and their function in vasculogenesis and angiogenesis

Differentiation of endothelial precursor cells need specific molecules for adhesion and migration during vasculogenesis, sprouting of vessels as well as cell recruitment in angiogenesis but also signal receptors for proliferation, survival and apoptosis. Integrins are a family of heterodimeric transmembrane cell surface glycoprotein adhesion molecules. They consist of one out of 18 α and one out of 8 β subunit, each containing a large extracellular domain, one α-helix crossing the cell membrane and a short cytoplasmic part. Integrins are involved in cell-cell contacts, cell-matrix adhesion and signal transduction. Mammalian cells express a large variety of different integrins on their cell surface mostly with overlapping functions. For fast cell response, signal transduction across the membrane takes place either in inside-out (e.g. intrinsic regulatory signals from cytoplasm control integrin function by transmitting signals) or outside-in (e.g. ligand binds to its receptor on the cell surface and sends signals to the cytoplasm) signaling through integrins (Qin, Vinogradova et al. 2004). The extracellular domain is connected to proteins of the extracellular matrix like fibronectin, fibrinogen, vitronectin, collagen, laminin, osteopontin and thrombospondin (table 1.10.1). The short cytoplasmic domain communicates with adaptor proteins like talin and paxillin connected to the cytoskeleton. Many of the

subunits of heterodimers have an embryonically lethal knockout phenotype. The heterodimer α5β1 is extremely important in angiogenesis. Genetic deficiency in integrin α5 results in embryonic lethality around day E10. Mice embryos suffer from a defects in normal development of the yolk sac and vessel formation resulting in vascular morphogenesis. Similar effects were reported of fibronectin-deficient embryos (Francis, Goh et al. 2002). Integrin β1 null mice have an even worse phenotype than α5 K.O. mice. They die earlier without any vascular network (Fassler and Meyer 1995). Specific integrin β1 K.O. in endothelial cells induced by the Tie-2-Cre system revealed sprouting and branching defects showing the importance for angiogenesis (Tanjore, Zeisberg et al. 2008). Integrin α4 subunit specific deletion in endothelial and hematopoietic cells leads to mobilization and results in increased fraction of bone marrow-derived circulating progenitor cells in the blood (Priestley, Ulyanova et al. 2007). Full knockout of α4 integrin shows failure in placentation, cardiac vessel growth and development of the outer layer of the heart wall (Yang, Rayburn et al. 1995). The absence of the subunit αv as part of the vitronectin receptor leads to 100% mortality within the first day after birth (Bader, Rayburn et al. 1998). Mice suffer from defects in cell interactions and cerebral hemorrhage (McCarty, Lacy-Hulbert et al. 2005). Interestingly, integrin β3- and β5-null mice are born with a normal vascular development and are fertile but have some defects in placenta and platelet aggregation (Hodivala-Dilke, McHugh et al. 1999). Surprisingly, these integrins show upregulation in sprouting angiogenesis (Max, Gerritsen et al. 1997). Because of the angiogenic involvement of integrins in cancer, some are targeted in clinical trials.

Table: 1.10.1: Some integrins and the receptors

Receptors	Integrins
Fibronectin	α5β1, α4β1, α4β7
Vitronectin	αvβ3, αvβ5
Collagen	α1β1, α2β1
Laminin	α3β1, α6β1, α6β4



http://anatomy.iupui.edu/courses/histo_D502/D502f04/lecture.f04/cell.f04/cellf04.html **modified**

1.10 Cbl-b, an E3 ubiquitin ligase

Ubiquitination is an important physiological process in modification, regulation of expression and recycling of damaged and redundant proteins. It is managed by the members of the large Ubiquitin-proteasome system in the nucleus and cytoplasm. Proteases of the E1, E2 and E3 family are very important in the proteasome system. Enzyme members of the E3 family catalyse the conjugation of ubiquitin molecules from E2 proteins to lysine residues of specific target molecules (Fig.1.11.1) resulting in mono-, multi- or polyubiquitylation (reviewed in (Ciechanover 2005)).

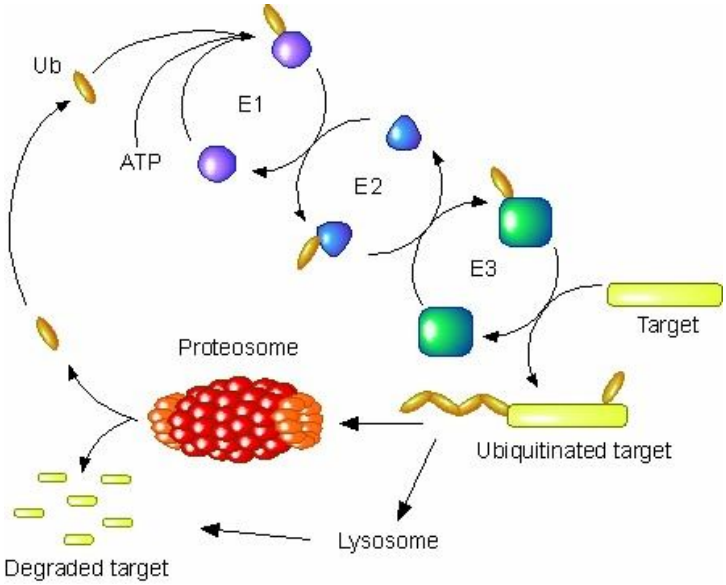


Figure 1.11.1: Ubiquitin-proteasome system

Three different types of enzymes are involved in the proteasomal pathway by tagging target proteins with ubiquitin degradation. www.med.upenn.edu/immun/PaulaM.OliverPhD.shtml

Polyubiquitylation mostly leads to protein degradation, whereas monoubiquitylation is involved in intracellular signalling processes (Hicke 2001). The Cbl family of the E3 ubiquitin ligases are key regulators of signal transduction of many cell surface receptors consequently regulate development and cell functions. Due to their discovery in retroviruses they are also known Casitas B-lineage (Cbl) lymphoma. In mammals, three homologues Cbl proteins have been identified so far: c-Cbl, Cbl-b and Cbl-c. Cbl proteins consist of a conserved N-terminal tyrosine-kinase-binding domain, a ring finger motif, a proline rich part and a C-terminal ubiquitin-associated domain with a leucine zipper motif which is missing in Cbl-c (Fig.1.11.2).

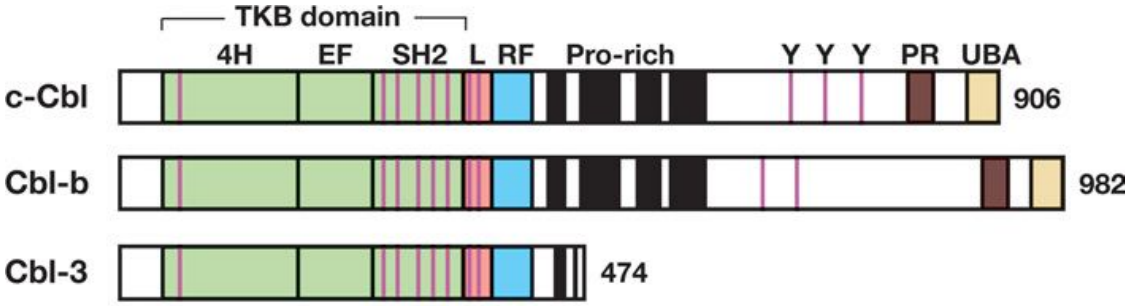


Figure 1.11.2: Cbl protein structure

Domain structure of c-Cbl, Cbl-b and Cbl-3 (≡Cbl-c) (modified after (Thien and Langdon 2005)).

c-Cbl and Cbl-b are adaptor proteins and play an important role in T cell immunity reflected in the phenotype of knockout mice (Bachmaier, Krawczyk et al. 2000) (Naramura, Jang et al. 2002). T cells are a subtype of the white blood cells, coming from the bone marrow and moving to the thymus for maturation. The process of T cells which present T cell receptors on their cell surface for recognizing antigens from non-self origin is called autoimmunity. Knockout of these two E3 enzymes leads to hypersensitivity of the T cell receptor tolerance and enhanced downstream signalling

independent of CD28 (Bachmaier, Krawczyk et al. 2000) (Chiang, Kole et al. 2000). Furthermore, Cbl-b is postulated to be a negative regulator of receptor clustering as well as raft aggregation (Krawczyk, Bachmaier et al. 2000). Cbl-b plays also a role in autoimmune diseases such as arthritis and diabetes (Yokoi, Komeda et al. 2002). Integrin-mediated cell signalling and adhesion by Rap1 activation is negatively regulated by Cbl-b (Shao, Elly et al. 2003). Cbl-b-deficient mice have an increased peripheral lymphocyte activation, organ infiltration by T and B lymphocytes and T cell proliferation with spontaneous autoimmunity (Bachmaier, Krawczyk et al. 2000) (Chiang, Kole et al. 2000). Moreover, c-Cbl and Cbl-b have regulatory effects in other cell lines. E3 enzymes, for example, control expression of stem cell factor receptor in mast cells (Zeng, Xu et al. 2005). Stem cell factor receptor belongs to the receptor tyrosine kinases (RTK) and is expressed on mast cells, hematopoietic stem cells and interstitial cells. Moreover, Cbl-b$^{-/-}$ mast cells produce higher levels of different cytokines like IL-6, TNFα and MCP-1 (Gustin, Thien et al. 2006) and filtrate into white adipose tissue (Hirasaka, Kohno et al. 2007).

1.11 Aim of this study

Endothelial precursor cells are required during embryonic and adult neovascularization. Previous studies already demonstrated that EPCs can be used for therapeutic enhancement of vascular repair in clinical trials. The vasculogenic potential of progenitor cells depends on an appropriate retention of the infused cells in the ischemic tissue. However, cell-autonomous mechanisms regulating the vascularisation by progenitor cells are not well understood. Beyond cell death signals, caspase enzymes regulate cell morphology and migration in many cell types. The isoform caspase-8 is involved in differentiation and proliferation and is essential for embryonic vasculogenesis in Caspase-8 deficient mice.

In this study, we first investigated the role of caspase inhibition for EPC survival and retention. These results turned our focus on caspase-8 as a critical protease for EPC function and maturation. In closer detail, we investigated the targets of caspase-8 involved in cell adhesion and migration

Besides surface molecules like integrins and CXCR4, we pointed our interest to the negative regulator of cell adhesion, Cbl-b, *in vitro* and *in vivo*.

2. Materials and Methods

2.1 Cells

The cells used in the different experiments are listed below (Table 2.1.1).

Table 2.1.1: Cells and their origin

Human/mouse cells	comment
EPC	Endothelial progenitor cells isolated from human peripheral blood from mixed donors
HUVEC	Pooled human umbilical vein endothelial cells (from Cambrex)
HMVEC	Human microvascular endothelial cells
HEK293	Human embryonic kidney epithelial cells (transformed cell line from Cambrex)
Mouse BMC	Bone marrow cells isolated from hind limbs of mice
Mouse spleen EPC	Endothelial progenitor cells isolated from spleen with Ficoll density gradient (protocol like EPC from peripheral blood)

2.2 Cell culture of HUVEC, HMVEC and HEK293T

HUVEC and HMVEC were grown in culture medium in flasks (Greiner) at 37°C, 5% CO_2 and 95% air humidity until the third passage. For passaging, cells were washed with phosphate buffer (PBS, Gibco) and trypsinated with 3 ml trypsin/EDTA

(Boehringer) until cells are almost detached. 10 ml EBM was used to stop enzyme reaction and resuspending the cells. For cell culture maintenance cells were seeded again in 250 ml flasks. For *ex vivo* experiments cell number was defined and cells were seeded with a density of 3.5-4.5 x 10^5 cells per 6 cm culture dishes. After 18-20 h of cultivation cells were used for experiments.

HEK293T cells were cultured in DMEM medium and passaged like HUVECs. Passages between two and three (HUVEC, HMVEC) and eight to fourteen (HEK293) were used for *ex vivo* experiments. The cell lines with the specific medium are listed below (Table 2.2.1).

Table 2.2.1: Cells and their specific medium

Cell type	Culture medium	Supplementary agents
EPC	Endothelial cell basal medium (EBM) from Lonza	20% fetal calf serum (FCS) (Boehringer); hEGF (10µg/mL), Hydrocortisone (1µg/mL), bovine brain extract (3µg/mL), Gentamycin Sulfat (50µg/mL), Amphotericin-B (50mg/mL) (= single quots Lonza)
HUVEC	Endothelial cell basal medium (EBM) from Lonza	10% fetal calf serum (FCS) (Boehringer); hEGF (10µg/mL), Hydrocortisone (1µg/mL), bovine brain extract (3µg/mL), Gentamycin Sulfat (50µg/mL), Amphotericin-B (50mg/mL) (= single quots Lonza)
HMVEC	Endothelial cell basal medium-2 (EBM-2) from Lonza	10% fetal calf serum (FCS) (Boehringer), single quots (Lonza)
HEK293	DMEM 4500 Glucose with Glutamax (from Gibco)	10% heat inactivated FCS, Penicillin/Streptomycin (500U/mL) (Gibco)
Mouse BMC	X-vivo 10 (Cambrex)	2% FCS
Mouse spleen EPC	Endothelial cell basal medium (EBM) from Lonza	20% fetal calf serum (FCS) (Boehringer); hEGF (10µg/mL), Hydrocortisone (1µg/mL), bovine brain extract (3µg/mL), Gentamycin Sulfat (50µg/mL) (= single quots Lonza)

2.3 Isolation and *ex vivo* endothelial differentiation of human endothelial progenitor cells (EPC)

Mononuclear cells (MNCs) were isolated by Ficoll (Biocoll density 1.077, Biochrom) density gradient centrifugation (800x g, 20 min, RT, no brake) from human peripheral blood buffy coats. Immediately after isolation, $8x10^6$ MNC/ml were plated on culture dishes or T-75 culture flasks (Greiner) precoated with fibronectin (10 ng/ml in PBS, Sigma) for 30 min and maintained in EBM complete (EBM + FCS + single quots) medium at 37°C, 5% CO_2 and 95% air humidity. After 3 days in culture, non-adherent cells were removed by thorough washing with PBS and adherent cells were incubated in fresh EBM complete medium and were used for following experiments e.g. with caspase inhibitors (Sigma) in different concentrations or cytokines like Interleukin-6 (5 and 10 ng/ml, Peprotech). Afterwards, cells were stained with 1,1'–dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (Dil-Ac-LDL, 2.4 µg/ml; Harbor Bio-Products) at 37°C for 1 h and fixed with 4% Formaldehyde. Further, growth factor release was measured by ELISA kits (Quantikine, R&D System) after stimulation. The concentration of inhibitors was adapted to each of the experiments. For longer incubation times the zIETD dose was decreased from 100 µM to 20 µM because the solvent DMSO showed some toxicity if incubated for prolonged time.

2.4 Isolation of EPCs from mouse spleen - EPC-assay

Spleens from mice were completely homogenised and carefully transfered on Ficoll (Biocoll density 1.077, Biochrom). After centrifugation at 800x g, 20 min, RT, no brake, mononuclear cells were collected and washed with PBS. After removing the supernatant cells were resuspend in EBM complete. $4x10^6$ cells were seeded into 24-well plates precoated with 1:100 diluted fibronectin (Sigma). After four to seven days at 37°C, 5% CO_2 and 95% air humidity cells were washed once with PBS and stained with 1,1'–dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (Dil-Ac-LDL) for 1 h at 37°C. After four days the number of

adherent EPCs was counted with a fluorescence microscope in 20x magnification (5 fields per view). To calculate the EPC colonies (one colony exists of 5 cells at least) the cells were incubated in EBM complete for three more days. Cells were fixed in 2% formalin solution and colonies were counted in 10x magnification.

2.5 Gene knockdown by RNA interference (siRNA-mediated)

Small interfering RNAs (oligonucleotides) were used for specific mRNA suppression in transfectable cells (

Table 2.5.1). HUVECs were grown to 60-70% confluence and transfected with GeneTrans II (MoBiTec). The hydrated Gene Trans II reagent was diluted in 0% EBM (EBM medium without FCS and single quots which contains growth factors and antibiotics). Specific siRNA or scrambled from 20 µM stock were diluted in DNA dilutent B (also supplied by MoBiTec). Both reagents were mixed well by pipetting several times and incubated at room temperature (RT) for 5 min. siRNA solution was added to the diluted Gene Trans II reagent and was incubated for additional 5 min to allow complex formation. In the meantime, HUVEC monolayers were washed two times with 0% EBM without antibiotics, FCS and supplements. 3 ml 0% EBM were added to the cells and the Gene Trans II/siRNA complexes were added dropwise. HUVEC were incubated at 37°C, 5% CO_2 and 95% air humidity for 4 h. Then, EBM medium with siRNA solution complex was removed and cells were washed with PBS. Fresh EBM complete was added to the cells. Expression of the target gene was analyzed after 24 and 48 h.

Table 2.5.1: Oligonucleotide sequences

siRNA (target gene)	sequence
Scramble	UCAAGAAGCCAAGGAUAAU
Cbl-b	CGGUUAAGUUGCACUCGAU

2.6 RNA isolation

The TRIzol reagent (Invitrogen) method was used to isolate whole mRNA from HUVECs and EPCs. Cells were washed carefully with PBS and directly lysed with an appropriate volume of TRIzol reagent (Table 2.6.1).

Table 2.6.1: Volumes of used Trizol and Chloroform for defined number of cells

Number cell/tube	Volume of TRIzol	Volume of chloroform
1 x10^6-5 x10^6	1 ml	0.2 ml
1 x10^5-1 x10^6	0.8 ml	0.160 ml
≤ 1 x10^6	0.4 ml	0.080 ml

The cells were homogenised in the reagent by pipetting until all cell components were dissolved. After 5 min of homogenisation at room temperature, chloroform was added as indicated (Table 2.6.1). After shaking the tubes for 15 seconds and incubation for additional 3 min, the suspension was centrifuged at 12.000x g, 4°C for 15 min. The colourless upper aqueous phase (containing RNA) was transfered in a new tube. The approximate volume was defined, 0.8 times of isopropyl alcohol was added and shaken vigorously by hand. After 10 min of incubation at room temperature, samples were centrifuged at 14.000x g, 4°C for 15 min. The supernatant was well removed and the pellet washed with 75% ethanol. After spinning again and discarding the supernatant, the pellet was air-dried for 10 minutes. Afterwards the RNA pellet was dissolved in 15-20 µL RNAse-free H_20.

2.7 Oligonucleotide microarrays (Affymetrix, Inc.)

RNA from EPCs, HUVECs, mononuclear cells and $CD34^+$ cells were send to the Affymetrix Inc. for RNA expression analysis. Ten microgram of total RNA was hybridized to the HG-U95Av2 microarray (Affymetrix, Inc.). The standard protocol

used for sample preparation and microarray processing is available from Affymetrix. Expression data were analyzed using Microarray Suite version 5.0 (Affymetrix, Inc.) and GeneSpring version 4.2 (Silicon Genetics).

2.8 RT-PCR

RNA concentration was determined in UV-light at 260 nm. For RT-PCR 2 µg of RNA were first reverse transcribed into cDNA. Afterwards, mRNA was reverse transcribed by M-MLV Reverse Transcriptase (Invitrogen). GAPDH expression was used as loading control. For semiquantitative RT-PCR, conventional PCR was done with specific primers (Table 2.8.1 and 2.8.2) against the target genes and afterwards, DNA was separated in agarose gel to visualise the amount of DNA.

Table 2.8.1: Human primer sequences

RT-PCR	5` →3` sense sequence	5` →3` antisense sequence
Human Caspase-8	CTGTGCCCAAATCAACAAGAGC	CTGTCCATCAGTGCCATAGATG
Human Cbl-b	GGAGAAGACTTGGAAGCTCATG	CATGAAGGCACTGTCTGAATAC
GAPDH	TCACCATCTTCCAGGAGCGAGATC	GAGACCACCTGGTGCTCAGTGTAG

Table 2.8.2: Murine primers for genotyping

RT-PCR genotyping	Allele	5`→3`sense sequence	5`→3`antisense sequence
Caspase-8 conditional knockout mice	Wildtype, knockout, floxed and deleted allel	TAGCCTCTTTGGGGTTGTTCTACTG	
	Deleted allel		GCGAACACGCCGTGTTTCAAGGGC
	Wildtype, knockout and floxed allel		CGCGGTCGACTTATCAAGAGGTAGAAGAGCTGTAAC
Cbl-b knockout mice	Wildtype	CATCTCAGTGTTTGAATTTG	GGAAAAATATTAGTTACAACTGG
	Deleted	ATGCCTGCTCTTTACTGAAG	GGAAAAATATTAGTTACAACTGG

2.9 Plasmid transfection in HUVEC and HEK293 using Superfect (Qiagen)

HUVEC and HEK293 were transfected one day after seeding with 80% confluence. First, 3 µg plasmid DNA and 18 µl Superfect were mixed in 150 µl 0% EBM medium by vortexing. During the 10 min of DNA-Superfect incubation, cells were washed with 0% EBM medium. Then, 1.2 ml of EBM 10% with supplements was added to the transfection mix. After removing the starving medium, the transfection solution was equally added to the cells. After 3 h of incubation in an incubator (37°C, 5% CO_2, 95% air humidity), solution was removed and 3 ml of EBM 10% complete was added. Cells were harvested after 48 h by lysis of the cells.

Table 2.9.1: Plasmids used in the experiments

Plasmid	Vector	Insert side	Selection	Total size
Caspase-8 (Flice)	pcDNA3.1(-) (Invitrogen)	BamH I/ Hind III	Ampicillin	~ 1440 bp
Cbl-b wt-HA	pCEFL	Hind III/ Kpn I	Ampicillin	~ 2200 bp
Cbl-b N 1/2-HA	pCEFL	Hind III/ BamH I	Ampicillin	~ 1480 bp
Cbl-b N 1/3-HA	pCEFL	Hind III/ BamH I	Ampicillin	~ 1047 bp
Cbl-b C 2/3-HA	pCEFL	Hind III/ Kpn I	Ampicillin	~ 1833 bp

2.10 Lentivirus-transduction in HUVEC and EPC

HUVECs and EPCs were seeded in 12-well plates to 80% density ($1x10^5$ cells/well). The Multiplicity of Infection (MOI) is the number of vector units used to transduce a single cell. The used MOI is five; consequently the number of vector transducing units per cell is 5. The vector units provided by Sigma-Aldrich for caspase-8 differ between $1x10^7$ and $1x10^8$ transducing units (TU) per milliliter. Each well was transduced with $5x10^5$ lentiviral particles supported by polybrene (8 µg/ml), a cationic polymer which increases the efficiency of shRNA uptake into the cells. RNA isolation was done with the RNAeasy micro kit (Qiagen), followed by reverse transcription PCR.

2.11 Protein isolation from whole cells

For protein isolation cells were washed with ice cold PBS and lysed in lysis buffer (20 mmol/l Tris [pH 7.4], 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 1% Triton, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l ß-glycerophosphate, 1 mmol/l Na_3VO_4, 1 µg/ml leupeptin, and 1 mmol/l phenylmethylsulfonyl fluoride) containing

protease inhibitors (Roche) for 20 min on ice. Insoluble cell components were separated from the cell lysate by centrifugation for 15 min at 20000x g (4°C). Protein concentration was measured with Bradford Reagent.

2.12 Immunoprecipitation

After Cbl-b protein overexpression, cells were lysed in buffer (for procedure see above 2.11.). For preclearing the samples (500 µg), they were incubated with protein A/G Plus-Agarose (Santa Cruz) for 30 min at 4°C followed by cold high speed centrifugation. Then, the specific antibody (anti-HA tag: Roche) was added to the supernatant with rotation at 4°C over night. Subsequently, 20 µl protein A/G Plus-Agarose was added for 1 h at 4°C. Finally, lysates were washed carefully with lysis buffer. Immunoprecipitated Cbl-b was incubated with 2 units of recombinant caspase-8 (Chemicon). At the end, the precipitates were mixed with loading dye and were boiled for 5 min before the cell proteins were separated in gel electrophoresis.

2.13 Colorimetric analyses of protein concentration according to Bradford

For protein concentration analysis 1 µl protein lysate was mixed into 799 µl H_2O and 200 µl Bradford reagent and incubated for 5 min at room temperature. The absorption of protein was measured in a spectral photometer at 596 nm against a reference. Protein concentration was calculated on the basis of a bovine serum albumin standard curve.

2.14 SDS-polyacrylamid gel electrophoresis (SDS-PAGE)

To separate proteins, 4x loading buffer (250 mM Tris/HCl ph 6.8, 8% SDS, 40% Glycerin, 0.04% Bromphenolblau, 200 mM DTT) was added to a defined protein amount. The solution was boiled for 5 min at 100°C to denature proteins. To separate proteins according to their electrophoretic mobility, protein homogenisates were used

in a discontinuous sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-Page) after Laemmli. First, the protein solution was filled in the stacking gel (0.5 M Tris/HCl, pH 6.8; 0.4% SDS) to concentrate the protein. The resolving gel is a small pore size polyacrylamide gel ranging between 7 – 15% (0.5 M Tris/HCl, pH 6.8; 0.4% SDS) depending on the expected protein size. Separation was done with 80 V in the stacking gel and 120 V in the resolving gel using running buffer (0.25 M Tris, 1.92 M Glycin and 1% SDS) in the Mini Protean electrophoretic system (BIO-RAD). Molecular weight was detected by a standard protein marker (rainbow marker, Amersham).

2.15 Wet-blot method for specific protein detection (Western blot)

The Western blot method (also called immunoblot) is used to transfer proteins from the resolving gel onto polyvinylidenfluorid (PVDF) membranes (Millipore) by an electric field. First the PVDF membrane was activated in methanol for 1 minute and then rinsed in H_2O for 2 min. Then, 2 sponge pads and 6 papers of whatman were preincubated in 1x wet blot buffer (0.05 M Tris, 0.038 M Glycin, 0.1% SDS, 20% Methanol). A sponge pad, Whatman paper (3 x), nitrocellulose membrane, gel, again Whatman and sponge pad were built-up to a stack without air bubbles. The stack was put vertically in a wet blot chamber (BIO-RAD) filled with 1x wet blot buffer. The transfer ran at 20 W for 1.5-2 hours at 4°C. Transfered proteins were detected in an antibody-enzyme reaction. The PVDF membrane was probed with specific antibodies to detect the target protein. First, the membrane was blocked with TBS/Tween (50 mM Tris/HCl, ph 8, 150 mM NaCl, 2.5 mM KCl, 0.1% Tween-20) containing 3% non-fat dry milk (Töpfer) or 3% bovine serum albumin (Merck) for 1 h gently shaking (Rotator, IKA-Labortechnik). Blocking the membrane is necessary to prevent non-specific binding of the antibody to the membrane. Subsequently, the specific target antibody in a tested dilution was added to the membrane for 2 h at RT or over night at 4°C. After washing the membrane 3x with TBS-T, a species-specific (e.g. anti-mouse, anti-goat or anti-rabbit) horseradish peroxidase-linked secondary antibody was used in conjunction with a chemiluminescent agent. After washing the

membrane again 3x with TBS-T, the PVDF membrane was incubated with a substrate (Amersham) which luminesces when exposed to the conjugated enzyme on the secondary antibody. For detection of the signal, x-ray films (Hyperfilm-ECL, Amersham) were exposed to the membrane for various time points. The following table contains the used antibodies for Western Blot analysis (Table 2.15.1).

Table 2.15.1: Western Blot antibodies

First Antibody (target protein)	Dilution	Blocking	Company	Secondary antibody
Caspase-8	1:1000	5% milk	Immunotech	Anti-mouse-HRP
Cbl-b	1:1000	3% BSA	Santa Cruz	Anti-rabbit-HRP
Integrin alpha5	1:500	3% BSA	Chemicon	Anti-rabbit-HRP
Integrin beta1	1:2500	3% BSA	Chemicon	Anti-mouse-HRP
Anti-ERK 1/2	1:1000	3% BSA	Cell Signaling	Anti-rabbit-HRP
Anti-HA	1:500	3% BSA	Roche	Anti-mouse-HRP

2.16 Adhesion assays

Ninety-six-well plates were coated overnight at 4°C with 5 µg/ml human fibronectin (Roche), ICAM-1 or Collagen I in coating buffer (150 mM NaCl, 20 mM Tris HCl, 2 mM $MgCl_2$, pH 9.0) and then blocked for one hour at room temperature with 3% (w/v) Polyvinylpyrrolidone (PVP) in PBS. *Ex vivo* expanded human EPCs were incubated for 40 min at 37°C in the presence of zIETD (100 µM) and afterwards stained with 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM). After detachment with trypsin all cells were resuspended in RPMI

1640 with 0.05% BSA. Subsequently, EPCs were counted and $1.0x10^5$ cells/100 µL were seeded into fibronectin coated wells for 40 min at 37°C. Adherent EPC were quantified in triplicates with a fluorescence plate reader (Fluostat, BMG Lab Technologies) by washing of non-adhering cells with RPMI 1640 in several steps. The input was calculated with the plate reader before the first wash.

2.17 EPC and HUVEC migration assay

To determine the migration capacity of progenitor cells or transfected HUVECs, *ex vivo* expanded and for 6 h with zIETD preincubated human EPCs or HUVECs were resuspended in serum-free RPMI 1640 (Gibco) containing 0.05% BSA (Sigma, Germany). AT the previous day transwell membranes with 5 µm pore size (Costar) were coated on both sides with fibronectin (2.5 µg/mL; Roche) overnight at 4°C. Then, EPC ($1.5x10^5$ cells/well) or HUVECs ($0.5x10^5$ cells/well) were incubated in the upper chamber at 37°C in 5% CO_2 and allowed to migrate for 16 to 18 h (5 h HUVECs) toward the lower chamber in the presence or absence of 100 ng/mL human SDF1α (Peprotech). Cells remaining on the upper surface of the transwell membranes were mechanically removed and cells that had migrated to the lower surface were fixed with 4% formaldehyde. For quantification cell nuclei were stained with 4`,6-diamidinophenylidole (DAPI). Migrating cells on the bottom of the chamber were counted in 5 random microscopic fields using a fluorescence microscope (Axiovert 100, Carl Zeiss).

2.18 “Scratch wound” assay

Pooled human umbilical vein endothelial cells (HUVEC) were cultured until the third passage. After detachment with trypsin, $3.5x10^5$ cells were grown in 6 cm culture dishes for at least 20 h. Afterwards cells were stimulated with 100 µM inhibitors (e.g. zIETD, zDQMD, zVAD) for 16 h. The next day, the cell monolayer was scraped with a sterile cell scraper to create a cell-free zone (width 14 mm). Cells were washed with

medium and treated with inhibitors again as indicated for additional 24 h. Endothelial-cell migration was quantified by measuring the width of the cell-free zone (distance between the edges of the injured monolayer) at the time of injury and after 24 h of cultivation using a computer-assisted microscope (Zeiss) at 5 distinct positions (every 5 mm).

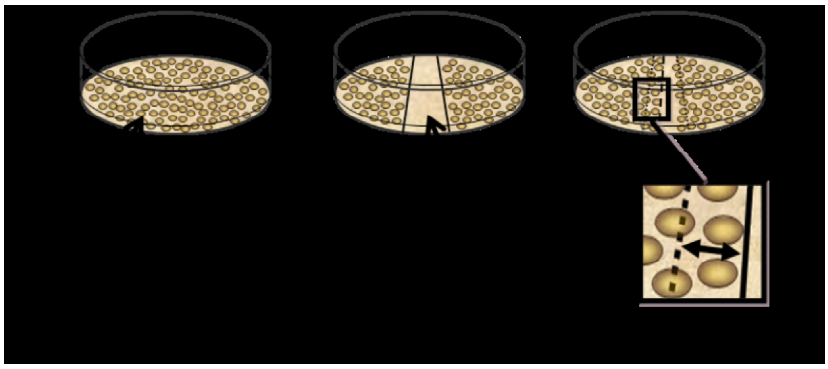


Figure 2.18.1: Schematic illustration of the scratch wound experiments.

2.19 Spheroid assay

In this three dimensional assay, cells are characterized by their sprouting ability in a methocoel-collagen gel. Transfected HUVECs were detached by adding Trypsin/EDTA. EBM medium with 10% FCS and supplements was used to stop the reaction. After pelletization, 48.000 cells were suspended in 12 ml EBM (10% FCS and supplements) – methocoel-mix (80 to 20%). With a multistepper device, 100 µl from this cell suspension was transfered into the cavity of a 96-well plate with U-bottoms. After 24 h in the incubator, round spheroids were harvested and overlaid with 80% methocoel/ 20% FCS solution mix. After adding a collagen solution (pH 7.4) and carefully mixing both solutions, the spheroid-collagen mix was transfered into the cavity of 24-well plate. After polymerization of the collagen gel at 37°C for 30 min, 100 µl of culture medium were added on top of the gel. After 24 h of incubation at 37°C and 5% CO_2 sprouted spheroids were fixed with 10% formaldehyde.

2.20 Detection of proteins in whole cells by fluorescence microscopy

After three days of isolation, attached EPCs were cleared by washing from non-attached cells. Cells were stimulated with caspase-8 inhibitor for the indicated period of time in cell culture medium. After carefully removing any solution, cells were fixed and treated with saponine for permeabilization. Staining with specific antibodies (caspase-8, von-Willebrand factor, integrin α5, anti-nuc) and DNA (TOPRO, DAPI) markers was followed by fluorescence microscopy.

2.21 Fluorescence activated cell sorting (FACS) of EPCs and mice bone marrow cells

Cells were harvested with EDTA or directly isolated from hind limb bone of mice and were incubated with specific antibodies (Table 2.19.1) for 30 min at 4°C or RT followed by 4% formaldehyde fixation. Finally, immunofluorescence labeled cells were analyzed by quantitative flow cytometry using FACS CantoII (Becton Dickinson) and Diva Software.

Table 2.21.1: Specific antibodies for human and mouse species

Antibody	Company	Species
CD117 (c-Kit)	BD	mouse
CD11a,b,c	BD	human
CD18	BD	human
CD184 (CXCR4)	BD	mouse
CD184 (CXCR4)	BD	human
CD29 (integrin b1)	BD	human
CD49d (integrin alpha4)	BD	mouse
CD49e (integrin alpha5)	Immunotech	human
CD49e (integrin alpha5)	R&D Systems	mouse
CD45	BD	human
KDR (Flk-1, VEGF-R2)	BD	mouse
KDR (Flk-1, VEGF-R2)	R&D Systems	human
Lectin	Abcam	human
Sca1	eBioscience	mouse
vWF	Abcam	human

2.22 Apoptotic measurement of endothelial precursor cells with Annexin V (BD Pharmingen)

EPCs were starved with 0% EBM and stimulated with 50 ng/ml TNFα (Sigma). Cells which undergo apoptosis expose phosphatidyl serine from the inner membrane to the outside of the cell surface. Suspension cells or adherent cells (detached by trypsin) were washed twice with the supplied binding buffer. After cold centrifugation, cell

pellets were resuspended in 50 µl binding buffer. FITC-annexin V antibody was added and the cells were incubated for 15 min in the dark at room temperature. Annexin V specifically binds to negatively charged phospholipids like the phosphatidyl serine in the plasma membrane. Afterwards, percentage of apoptotic cells was detected by fluorescence activated cell sorting (FACS).

2.23 Caspase activity analysis

Caspase activity was detected under normal cell culture conditions or after specific inhibitor treatment with apoptotic pushing reagents. Under normal conditions, cells were washed once with cold PBS and protein extract was produced using provided lysis buffer (Kit Caspase-3-Chemicon, Kit Caspase-8-Sigma-Aldrich). After measurement of protein concentration, 100 µg were used in the colorimetric assay kit. Cell lysates were incubated together with Acetyl-Ile-Glu-Thr-Asp-p-Nitroaniline (Ac-IETD-pNA) or Acetyl-Asp-Glu-Val-Asp-p-nitroaniline (Ac-DEVD-pNA) as caspase substrates for 3 h to detect hydrolysis activity of caspase-8 and -3. Detection of the absorption the released chromophore p-Nitroaniline moiety was done by a microplate reader at 405 nm.

For caspase-8 specificity, cells were washed with PBS and incubated for 5 h with EBM 0% without supplements and two hours with TNFα (50 ng/ml) to induce stronger caspase-8 activity. Further steps were done like in the protocol for normal treatment.

2.24 Murine ischemic hind limb model and BMC transplantation

The effect of injected mouse BMCs or human *ex vivo* expanded EPCs on ischemia-induced neovascularization was investigated in a murine model of hind limb ischemia. $1x10^6$ bone marrow mononuclear cells or EPCs were transplanted intravenously in the tail of mice one day after causing hind limb ischemia. Two weeks later, the morphology of the limb was determined (toe necrosis rate) and the blood flow ratio of the ischemic to the normal leg was measured using a laser Doppler

blood flow meter (Laser Doppler Perfusion Imager System, moorLDI-Mark 2). The perfusion of the ischemic and non-ischemic limb was calculated on the basis of colored histogram pixels. To minimize variables including ambient light and temperature and to maintain a constant body temperature, mice were exposed to infrared light for 10 min before laser Doppler scans. During the scan, mice were lying with their back on a heating pad with their legs stretched and fixed. The calculated perfusion was expressed as the ratio of ischemic to non-ischemic hind limb perfusion.

2.25 Immunohistochemistry

For morphological analysis, 10 µm frozen sections of the adductor and semi membranous muscles were used. Frozen sections were thawed for 30 min and incubated in ice-cold acetone for 2 min. Myocyte membranes were stained using anti-laminin (Abcam) followed by anti-rabbit-Ig Alexa Fluor 488 or 647 (Molecular Probes) and anti-CD31 (BD). After washing 3x with PBS sections were capped with glass cover slides.

For detecting incorporation of human EPCs into hind limb ischemic tissue, frozen sections were thawed for 30 min at RT and fixed with 4% PFA for 10 min followed by permeabilization with 0.2% Saponin/PBS for 15 minutes. Mouse tissue was blocked with 2% mouse serum for 1 h at RT. Sections were washed with PBS/2% NaCl/0.05% Tween20 and stained against human-nuclei-antigen (1:100; Chemicon) for 60 min in blocking solution followed by mounting with DAPI medium.

2.26 Matrigel plug assay

For analysis of vascularization capacity of EPCs *in vivo*, we subcutaneously injected 500 µl of growth factor reduced matrigel (BD Bioscience) with 3 µl Heparin-Sodium 25000 (Ratiopharm) on the back of nude mice. $1x10^6$ e*x vivo* expanded and pretreated cells were transplanted intravenously in the tail of the mice. After 7 days

the matrigel plugs were harvested for hemoglobin concentration measurements. Plugs were homogenized and matrigel lysates were used in the Drabkin assay (Sigma-Aldrich) Stock solutions of hemoglobin were used to generate a standard curve. Results are expressed relative to total protein in the supernatant.

Alternatively, the matrigel plugs were harvested after 7 days and embedded into paraffin. Sections were stained using a Cy3-labeled mouse monoclonal antibody for smooth muscle actin positive cells (Sigma) as well as Isolectin B4 (Vector) and scanned with a confocal microscope.

2.27 Statistical analysis

The record data are expressed as mean ± SEM. Comparisons between groups were analyzed by t-test or ANOVA (post hoc test: LSD= least significant difference) for experiments with more than two subgroups (SPSS software). P values <0.05 were considered as statistically significant. Numbers of independently performed experiments are mentioned at n.

3. Results

3.1 Caspases in endothelial precursor cells

Human peripheral blood mononuclear cells were isolated by ficoll density gradient centrifugation and incubated under endothelial differentiation conditions (endothelial basal medium (Lonza) with 20% FCS and single quots (Lonza) containing endothelial growth factors, bovine brain extract, hydrocortisone, gentamycin sulfat and amphotericin-B) *ex vivo* to generate endothelial precursor cells (EPC). These *ex vivo* cultured pro-angiogenic EPCs express endothelial markers such as eNOS (Vasa, Fichtlscherer et al. 2001), KDR (VEGF-R2), CD31, CD105, vWF, VE-cadherin (Dimmeler, Aicher et al. 2001) (Urbich, Heeschen et al. 2005) and the myeloid and hematopoietic markers CD11 (Chavakis, Aicher et al. 2005) and CD45 (Urbich, Heeschen et al. 2003). Various cell surface markers are tested and are summarized in FACS analysis with the percental indication of expression on adherent EPC (Fig.3.1.1).

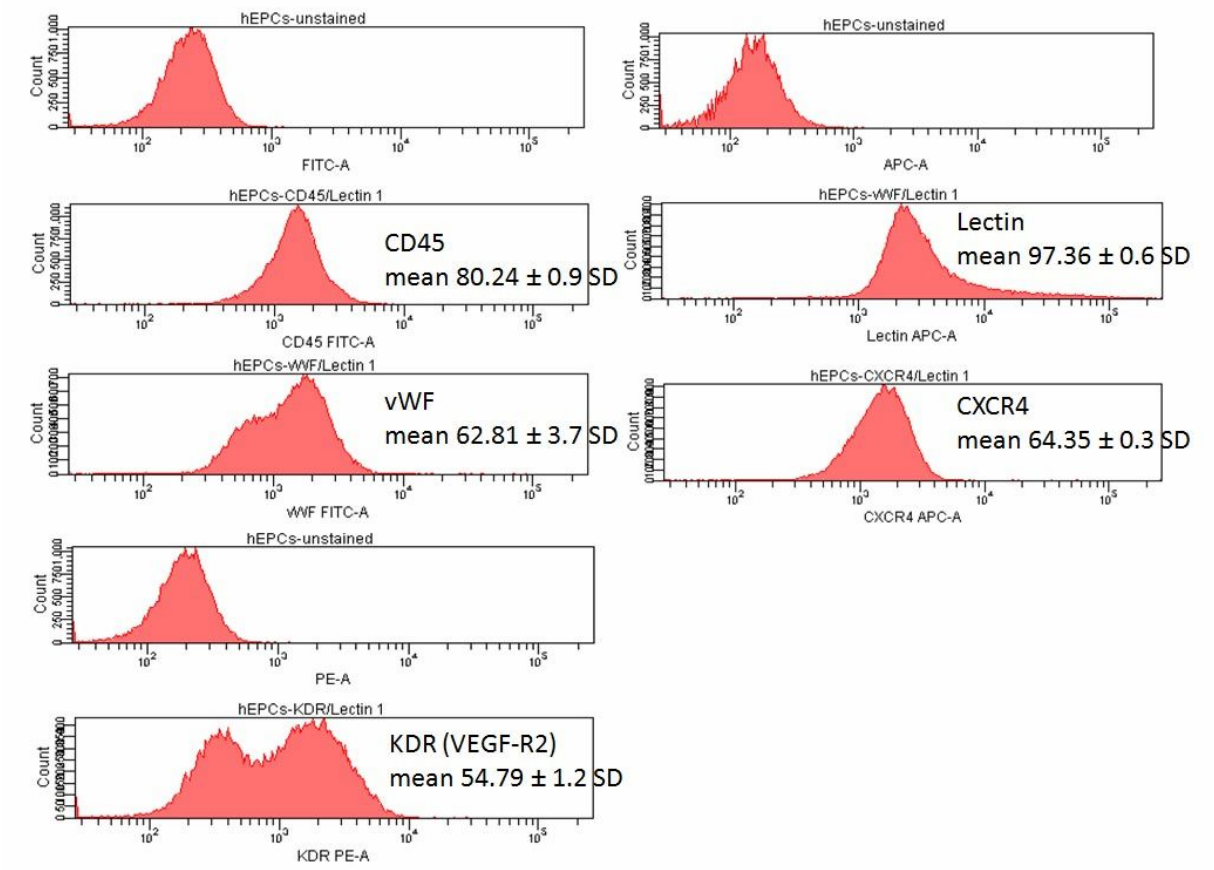


Figure 3.1.1: Cell surface expression of different marker on endothelial precursor cells

EPCs were tested for the cell surface expression of CD45, vWF, KDR, Lectin and CXCR4 by FACS analysis. Unstained cells were used as control. Almost all cells express lectin and CD45. More than the half of the measured cells expresses KDR, CXCR4 and vWF. The numbers indicate the percentage of total cells expressing the indicated markers.

Caspases have been originally described to be pro-apoptotic enzymes. Therefore, their inhibition may comprise a novel therapeutic strategy to improve cell survival of injected cells in ischemic tissues. However, unexpectedly addition of the broad-caspase inhibitor zVAD or the caspase-8 inhibitor zIETD during *ex vivo* cultivation unexpectedly abrogated the formation of fibronectin-adherent cells with endothelial cell characteristics (Fig.3.1.2a). Furthermore, the inhibitor zAEVD, which preferentially inhibits caspase-10, led to a partial inhibition of EPC formation, whereas the caspase-3 and -6 selective inhibitor zDQMD had no effect on EPCs.

The addition of the caspase-8 inhibitor zIETD to adherent EPCs growing on fibronectin during later stages (72 hours after isolation) of *ex vivo* cultivation caused a dose-dependent decrease in the number of adherent EPCs (Fig.3.1.2b).

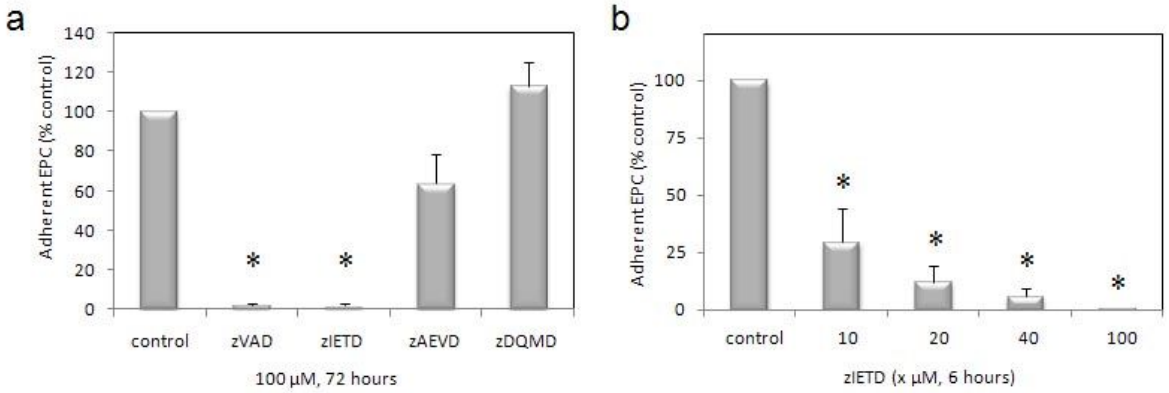


Figure 3.1.2: Effect of various selective caspase inhibitors on EPC formation and adherence

Mononuclear cells were isolated from human peripheral blood and cultured for 72 hours in the presence of different caspase inhibitors as indicated on the x-axis (100 µM). Formation of adherent precursor cells (a) was counted with a microscope after staining the cells with DiI-Ac-LDL and vWF. * $p<0.0001$ (b)The detection of the dose-dependent detachment of adherent precursor cells (72 hours of culturing and medium change) after incubation with zIETD for 6 hours was performed in the same way. * $p<0.01$; both experiments n=4, (mean ± SEM)

3.2 Caspase-8 expression in human cells

Having shown the strong effect of the broad-caspase inhibitor zVAD and the caspase-8 inhibitor on endothelial precursor cell formation, we analyzed Caspase-8 expression by western blotting in EPCs (Fig.3.2.1a, upper panel) and RT-PCR using specific primers in HUVEC, human peripheral blood mononuclear cells and endothelial precursor cells (Fig.3.2.1a, lower panel). Additional microscopic images revealed caspase-8 expression in the nucleus as well as in the cytoplasm

(Fig.3.2.1b). mRNA expression profiling indicates that caspase-8 is highly expressed in human CD34$^+$ cells, monocytes and fibronectin-adherent early EPCs compared to low expression in human umbilical vein endothelial cells (HUVEC) (Fig.3.2.1c).

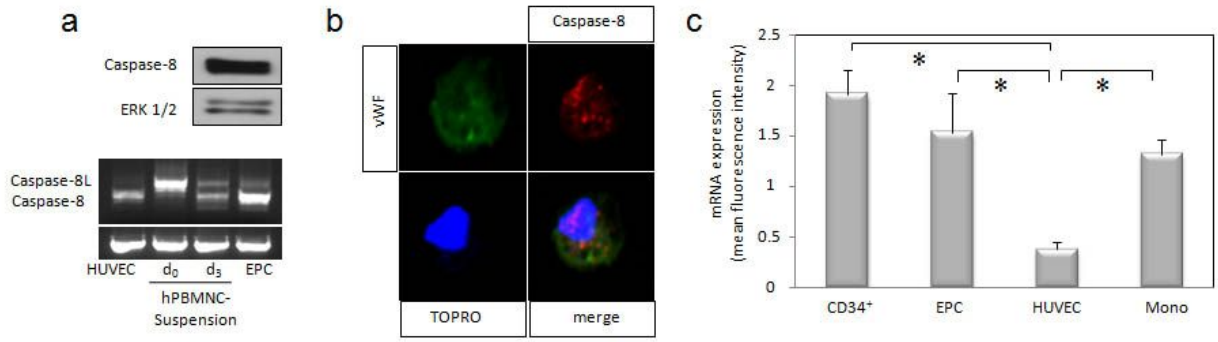


Figure 3.2.1: Caspase-8 detection in endothelial precursor cells

(a) Western Blot analysis to confirm protein expression in endothelial precursor cells and ERK 1/2 as loading control (upper panel). Semiquantitative RT-PCR was performed using RNA from HUVECs, human peripheral blood mononuclear cells (freshly isolated as well as cells grown in suspension for 72 hours) and outgrown (d3) endothelial precursor cells (lower panel). (b) Fluorescence images of outgrown EPCs by staining with antibodies against caspase-8, vWF (as endothelial marker) and TOPRO (dye for nuclear staining). (c) In a microarray, the mRNA expression level of caspase-8 was significantly higher in CD34$^+$ cells, monocytes (Mono) and endothelial precursor cells compared to HUVEC. * $p<0.05$ (mean ± SEM)

3.3 Caspase-8 activity is required for pro-angiogenic cell function *ex vivo*

In order to assess the effect of caspase-8 on functional abilities of EPCs, we performed adhesion and migration assays. After preincubation with zIETD or zDQMD for 4 h, *ex vivo* cultured EPCs were tested for the capacity to adhere to different matrices. Preincubation with zIETD but not zDQMD impaired the adhesion of EPCs to the major α5β1-integrin ligand fibronectin as well as to the β2-integrin ligand ICAM-1 and Collagen I matrices (Fig.3.3.1).

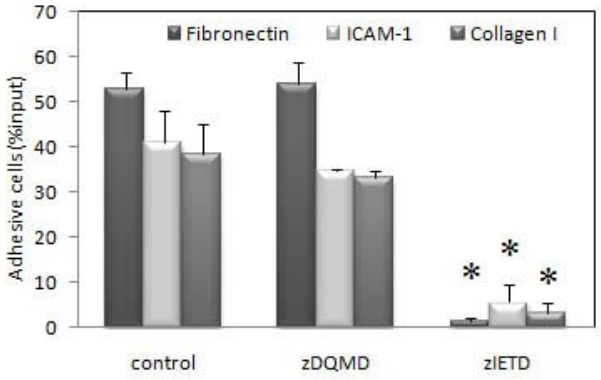


Figure 3.3.1: Adhesion capacity of EPCs subjected to caspase-8 inhibition

Endothelial precursor cells adhere to fibronectin, ICAM-1 and Collagen I following preincubation of the cells with zDQMD but not with zIETD (100 µM) for 4 hours. * $p<0.001$; $n=3$

Likewise in migration studies, zIETD pretreated EPCs were significantly inhibited in their capacity to move through a fibronectin-coated membran under basal conditions or with support of SDF-1α, VEGF or Interleukin-8 stimulation (Fig.3.3.2).

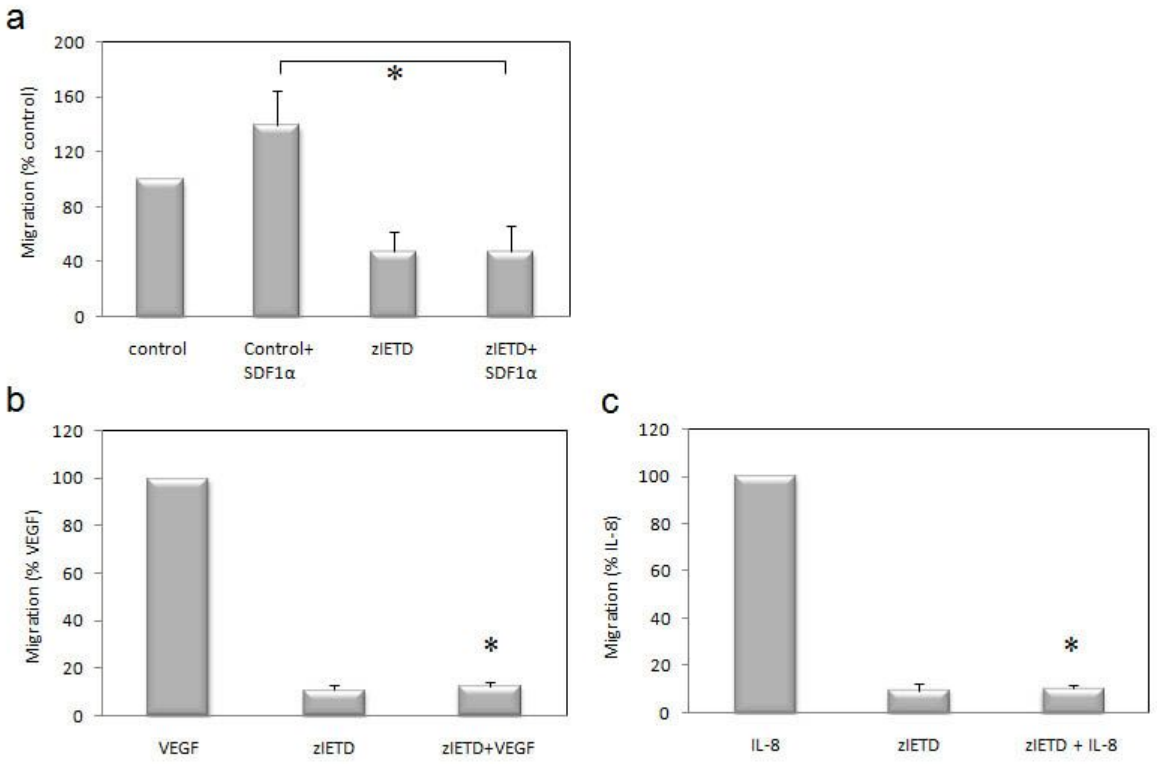


Figure 3.3.2: Adhesion capacity of EPCs subjected to caspase-8 inhibition

EPC migration was conducted in Boyden Chambers with SDF1α (100 ng/ml) (a), VEGF (50 ng/ml) (b) and IL-8 (50 ng/ml) (c) for 18 h after preincubation with zIETD for 6 h. * $p<0.05$; n=3 (mean ± SEM)

In contrast to EPCs, HUVECs remained adherent on uncoated plastic dishes even in the prolonged presence of high doses of caspase inhibitors (data not shown). In order to analyse the effect of caspase inhibition on the migratory capacity, HUVECs were first preincubated with different caspase inhibitors and subsequently subjected to a scratch wound assay. HUVEC migration was not affected by caspase inhibitors (Fig.3.3.3a). HMVEC migration was also not affected by caspase inhibitors tested in a boyden chamber migration (Fig.3.3.3b). These results suggest that the dependency of adhesion and migration on caspase activity is restricted to early circulating blood-derived cells.

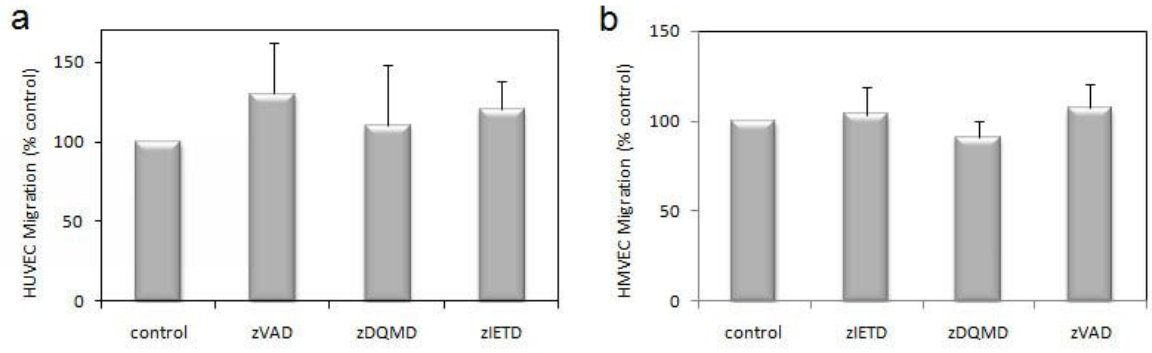


Figure 3.3.3: Migration of HUVEC in a scratch wound assay

(a) Pretreated (16 h with inhibitors) HUVECs migrated in a scratch wound assay for 24 hours in the presence of different caspase inhibitors (100 µM). (b) HMVEC were pretreated with different caspase inhibitors (100 µM) for 35 hours and migrated in boyden chamber for 5 hours. (both assays n=3, mean ± SEM, not significant)

These data indicate that caspase-8 is required for matrix adhesion and migration of endothelial precursor cells which are known for their potential in promoting neovascularization but not for these functions of mature endothelial cells.

3.4 Caspase-8 inhibition plays a role in cytokine release

To test whether caspase-8 is involved in cytokine secretion, we analyzed the culture medium of EPCs. Caspase-8 inhibition by zIETD profoundly disturbed the cytokine release by EPCs. The chemokine stromal cell-derived factor (SDF)-1α, ligand for the CXCR4 receptor, and the hepatocyte growth factor (HGF) were significantly reduced after 3 days of *ex vivo* cultivation compared to the solvent (Fig.3.4.1a,b). In contrast, the insulin-like-growth factor-1 (IGF-1) was slightly upregulated in EPCs cultured for 3 days in the presence of the caspase-8 inhibitor (Fig.3.4.1c).

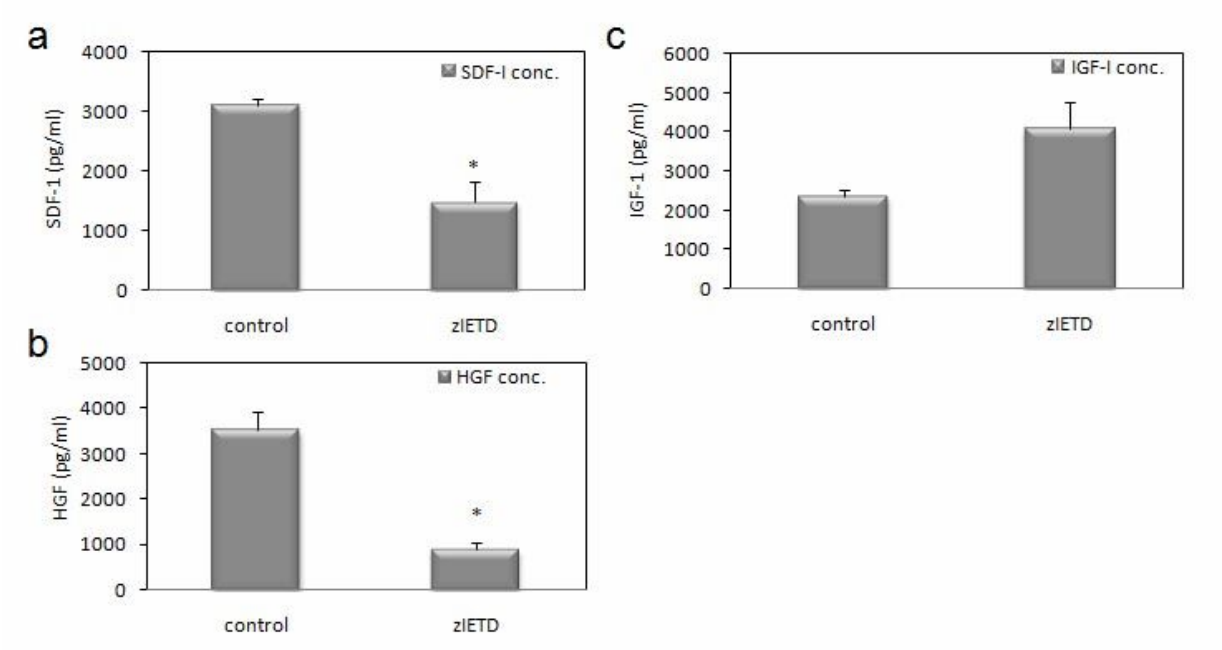


Figure 3.4.1: Growth factor release of caspase-8 inhibitor treated EPCs

(a,b) Significant reduction of growth factor SDF-1α and HGF release into the culture medium by endothelial precursor cells was observed after 3 days in *ex vivo* culture in the presence of caspase-8 inhibitor (100 µM). * $p<0.05$ (c) IGF-1 was slightly increased in the presence of zIETD in the culture medium compared to control cells. In both experiments, the growth factors were measured by ELISA. n=3 (mean ± SEM)

3.5 The activity of caspase-3 is not increased in adherent EPC

These previous data indicate that caspase-8 is required for formation and function of progenitor cells *ex vivo*. Disturbance of EPC formation and adhesion could be a consequence of apoptosis pathway induction. As a matter of fact, caspase-8 activity was detected in the majority of the cultured adherent cells (EPC day 3) under basal conditions compared to freshly isolated mononuclear cells and the suspension cells at day 3 (Fig.3.5.1a). Caspase-3 is a known target of caspase-8 to trigger apoptosis pathway induction (Boatright and Salvesen 2003). However, the higher caspase-8

activity in the adherent cells was not associated with an increase in caspase-3 activity (Fig.3.5.1b).

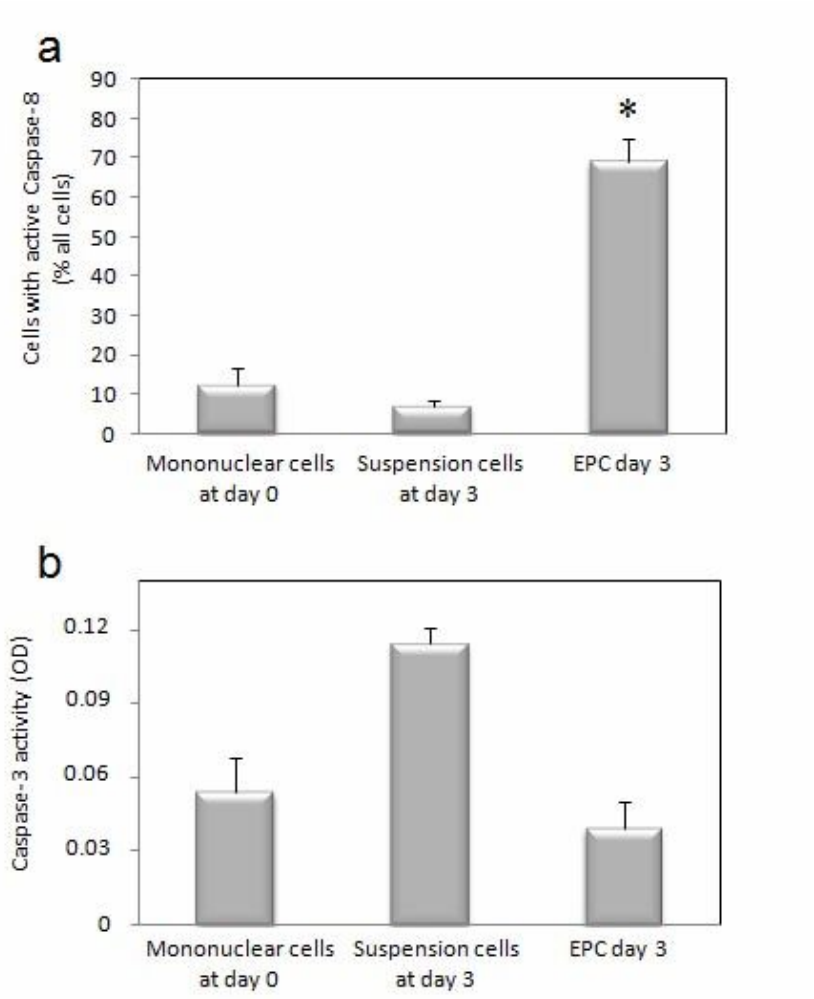


Figure 3.5.1: Caspase-8 and -3 activities in human endothelial precursor cells

Caspase-8 (a) and -3 (b) activities were measured in fresh isolated cells (mononuclear cells), suspension cells (72 hours after isolation) versus adherent EPCs at day 3 of *ex vivo* culture in a microplate reader. * $p<0.05$; mean ± SEM, both n=3.

These results support our idea that basal caspase-8 activation in adherent day 3 EPCs is to low to activate the apoptosis cascade pathway. In contrast, caspase-3 activity was observed in non-adherent mononuclear cells cultured for 72 hours resulting in a higher apoptosis rate and a short life-span (Fig.3.5.1b). These observations agree with apoptosis measurements, showing more apoptotic cells

within the suspension cell population compared to the fibronectin-adherent EPCs at day 3 of *ex vivo* culture (Fig.3.5.2).

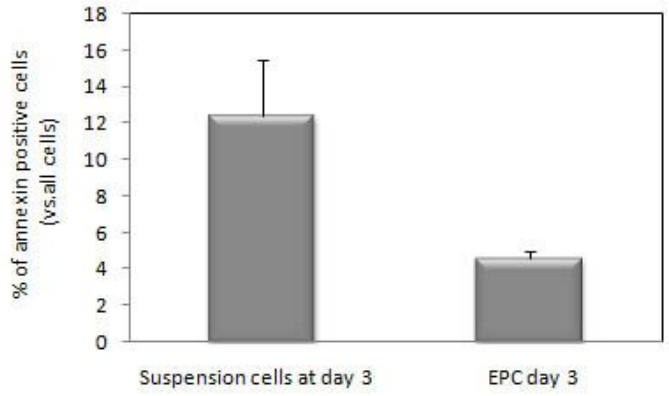


Figure 3.5.2: Measurement of apoptotic cells with Annexin V by flow cytometry

Suspension cells were compared with adherent cell at day 3 in *ex vivo* cell culture. Suspension cells were slightly higher in their amount of apoptotic cells compared to the adherent cells. The percentage of apoptotic cells of all measured cells is indicated in the graph. n=3, p=0.06

To prove that the caspase-8 inhibitor zIETD specifically affects caspase-8 activity, we used a caspase-8 colorimetric assay by Sigma®. EPCs were washed and incubated for 5 hours with 0% EBM medium without any growth factors and 3 hours with TNFα (50 ng/ml) to induce an increase in intracellular caspase-8 activity. Cell lysates were incubated with Acetyl-Ile-Glu-Thr-Asp-p-Nitroaniline (Ac-IETD-pNA), simulating a caspase-8 substrate, and additionally with zIETD inhibitor (100µM). Hydrolysis of the applied substrate results in the release of p-Nitroaniline (p-NA) measured at 405nm. The outcomes show a strong decrease of p-NA release after incubation of the cell lysate with the caspase-8 inhibitor zIETD (Fig.3.5.3) proving its specific inhibitory effect on caspase-8.

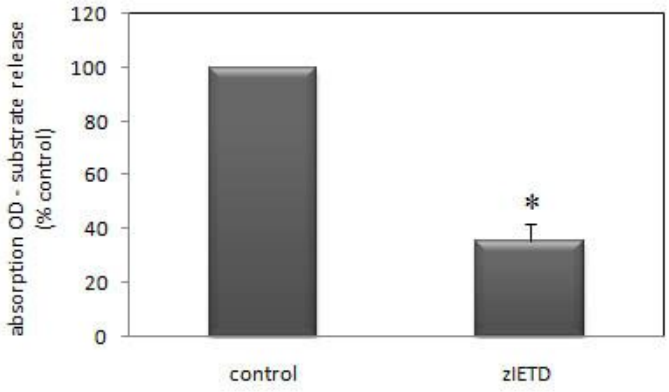


Figure 3.5.3: Caspase-8 activity assay

Cell lysates of adherent EPCs incubated for several hours with 0% EBM and TNFα were checked for their caspase-8 cleavage activity (control) as well as in the presence of caspase-8 inhibitor (zIETD). * $p<0.03$; $n=6$

3.6 *In vivo* incorporation and improvement of neovascularization by transplanted human outgrowing precursor cells is reduced by caspase-8 inhibition

In order to test, whether the observed reduction of adhesion and migration of cultured endothelial progenitor cells by the caspase-8 inhibitor leads to an impaired *in vivo* homing, cultured EPC were *ex vivo* pre-treated with a low dose of zIETD (20µM) for 18 hours and intravenously injected into nude mice 24 hours after hind limb ischemia. Harvesting of the muscles 72 hours after hind limb ischemia was followed by staining of frozen sections for human nuclear antigen indicative for engrafted human cells and with the DNA-fluorescence dye DAPI. Engrafted EPCs were detected in the ischemic tissue with fluorescence microscopy. Pretreatment with zIETD significantly reduced incorporation of infused cells 48 hours after administration (Fig.3.6.1a). Moreover, perfusion of matrigel plugs stimulated by EPC injection was significantly reduced after *ex vivo* pretreatment with zIETD (Fig.3.6.1b) indicating that cell autonomous caspase-8 activity indeed contributes to *in vivo* homing and improvement of neovascularization mediated by infused pro-angiogenic cells.

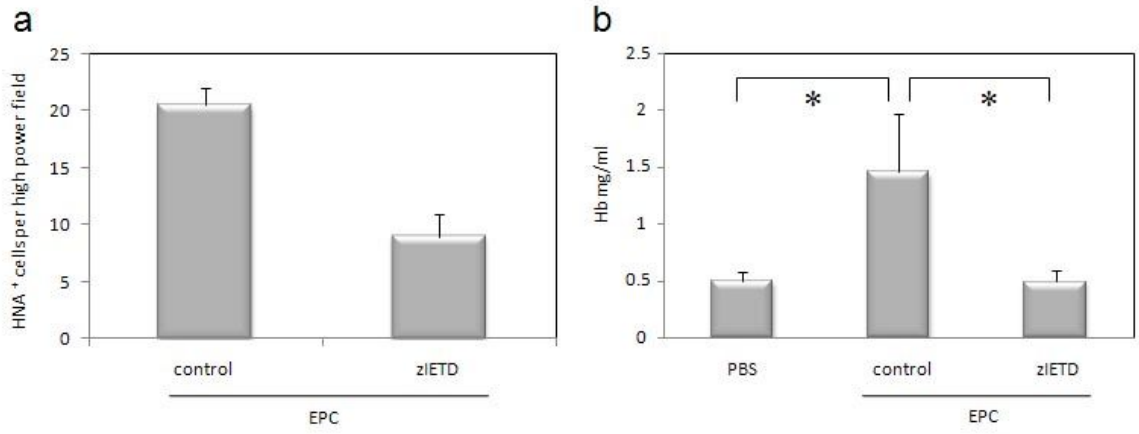


Figure 3.6.1: Effect of caspase-8 inhibition on vascularization *in vivo*

Intravenously admitted human EPCs were pretreated with caspase-8 inhibitor or solvent as control. (a) Numbers of human nuclear antigen^{+} (HNA) presenting cells were counted in frozen sections from murine hind limb tissue 2 days after ischemia. (b) The perfusion of matrigel was quantitatively assessed after transplantation of EPCs pretreated with caspase-8 inhibitor or solvent (* $p<0.05$; mean ± SEM, n=5 plugs per group).

Additional to the detection of engrafted human EPCs in ischemic tissue, post-ischemic neovascularization was observed by blood flow ratio two weeks after EPC transplantation between PBS-treated mice, EPC and zIETD-pretreated cells (Fig.3.6.2).

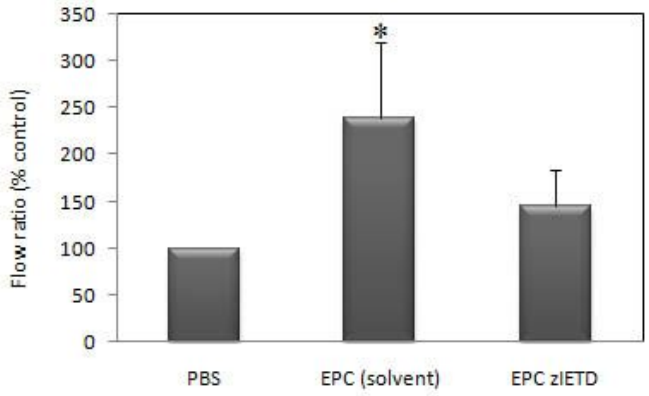


Figure 3.6.2: Effect of caspase-8 inhibition on neovascularization in ischemic tissue in vivo

Pretreated human EPCs (solvent vs. zIETD 20 µM for 18 h) were transplanted into nude mice following femoral artery ligation to induce ischemia. Enhancement of post-ischemic neovascularization by human EPCs measured by Laser Doppler given as the blood flow ratio of the ligated vs. unligated limb. Caspase-8 inhibition reduced the neovascularization capacity of EPCs; * $p<0.05$ vs. PBS-treated nude mice, mean ± SEM, n=4 per group.

3.7 Caspase-8 deficiency *in vivo* reduces functionality and vascularization capacity of bone marrow mononuclear cells

Our *ex vivo* studies give rise to the idea that caspase-8 is essential for EPCs. Caspase-8 deficient mice are embryonically lethal because of a reduced hematopoietic progenitor pool, limited heart development and hyperemia (Varfolomeev, Schuchmann et al. 1998). These results already claim a high non-apoptotic function of caspase-8 in vascularization and angiogenesis. Because of failed knockdown on mRNA level by lentivirus transduction in EPC compared to HUVECs (Fig.3.7.1), we were not able to continue *ex vivo* experiments combined with silencing of the caspase-8 gene.

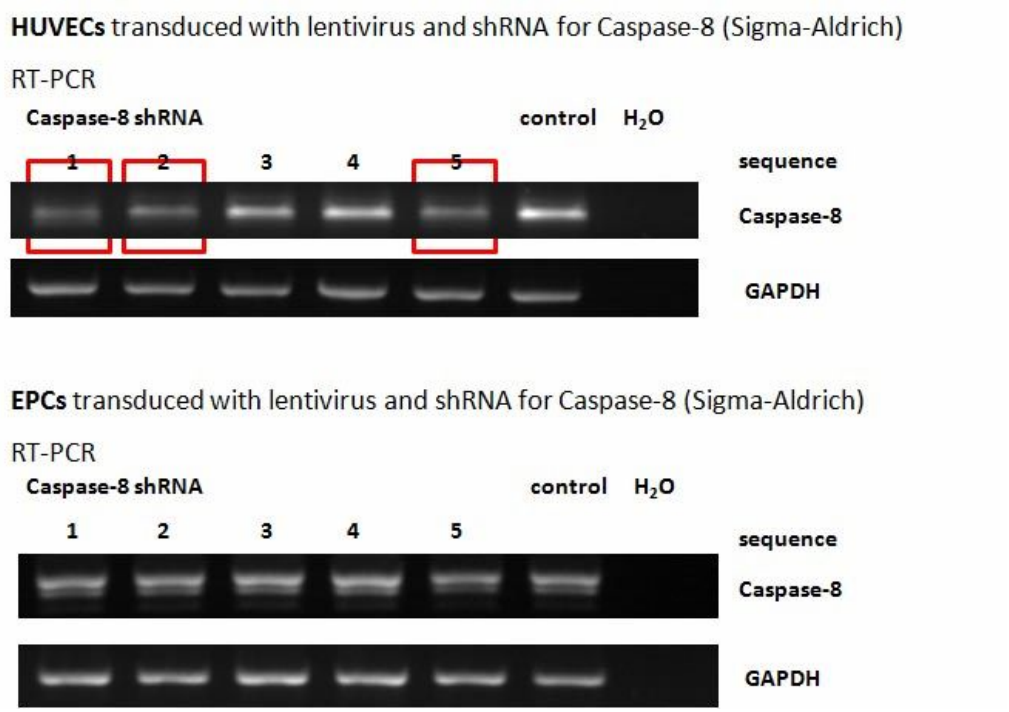


Figure 3.7.1: Caspase-8 knockdown by Lentivirus

Lentiviral transduction of a hairpin RNA against caspase-8 for silencing of the caspase-8 mRNA in HUVECs and EPCs. Various caspase-8 sequences (Sigma-Aldrich) showed different knowndown efficiency in HUVECs (red rectangle) compared to no reduction in EPCs by RT-PCR.

To elucidate the in vivo relevance of caspase-8, we used a mouse model in which exon 1 and 2 of the caspase-8 gene is conditionally deleted by the cyclization recombination enzyme (Cre) under control of the inducible Interferon-responsive myxovirus resistance-1 (Mx1) promoter. This promoter is silent in mice as long as the transcription levels is not induced by admission of interferon γ, or as in our case with synthetic double-stranded RNA poly I:C (pI-pC). One allel is knocked out (heterozygous) whereas the other is flanked by *loxP* recognition sites. The homozygous gene deletion is induced by Cre expression under the control of the Mx1 promoter in a tissue- and time-dependent manner (Kuhn, Schwenk et al. 1995). The Mx1 promoter targets hematopoietic precursor cell development resulting in reduced bone marrow functionality. Progenitor cells isolated from spleen, lymph node and liver after pI-pC injection showed reduced functionality (Kang, Ben-Moshe et al.

2004). Caspase-8 deletion in mice was checked with primers detecting the floxed, the non-floxed and the deleted allele (Kang, Ben-Moshe et al. 2004) (Fig.3.7.2).

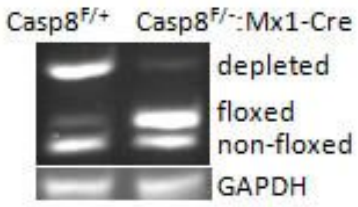


Figure 3.7.2: Caspase-8 deletion in mice

Caspase-8 deletion was checked by RT-PCR in bone marrow cells from mice to detect caspase-8 deletion. A primer set of three was used to check for deleted, floxed and non-floxed alleles. GAPDH primers were used as loading control.

Because of the limited blood volume in mice, it is impossible to isolate circulating mononuclear cells. For this reason, we used spleen mononuclear cells from different genetic caspase-8-deficient mice to compare the capacity to form fibronectin-adherent EPCs. The number of *ex vivo* adherent EPCs cultured for 4 days in endothelial basal medium was counted in a fluorescence microscope after uptake of Dil-Ac-LDL. Cells derived from caspase-8-deficient mice ($Casp8^{F/-}$:Mx1-Cre mice after pIpC treatment), showed a reduced capacity to form fibronectin-adherent EPCs compared to cells obtained from control animals (Fig.3.7.3).

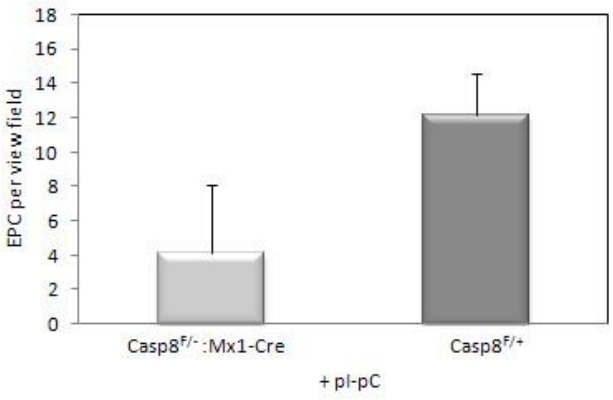


Figure 3.7.3: *Ex vivo* formation of mouse EPCs bearing Caspase-8 deletion.

Induced caspase-8 deletion in mice reduced the ability to form fibronectin-adherent splenic EPCs after 4 days in culture. Cells coming from mice with two functional caspase-8 alleles had a higher capacity in EPC formation, mean ± SEM, n≥3.

It has already been published that bone marrow cells improve neovascularization and angiogenesis in injured tissue (Assmus, Schachinger et al. 2002) (Schachinger, Erbs et al. 2006). To address whether caspase-8 gene deletion influences bone marrow cells in their capacity to improve post-ischemic neovascularization, we infused bone marrow cells into nude mice following femoral artery ligation in the right hind limb. Bone marrow mononuclear cells from conditional caspase-8-deficient mice had a significantly reduced capacity for enhancing limb perfusion when transplanted into mice in comparison with cells from mice with two functional caspase-8 alleles (Fig.3.7.4). These *ex vivo* and *in vivo* results demonstrate that caspase-8 deletion plays a crucial role in cell homeostasis and confirmed that caspase-8 activity is required for cell functionality and progenitor cell-mediated improvement of neovascularization *in vivo*.

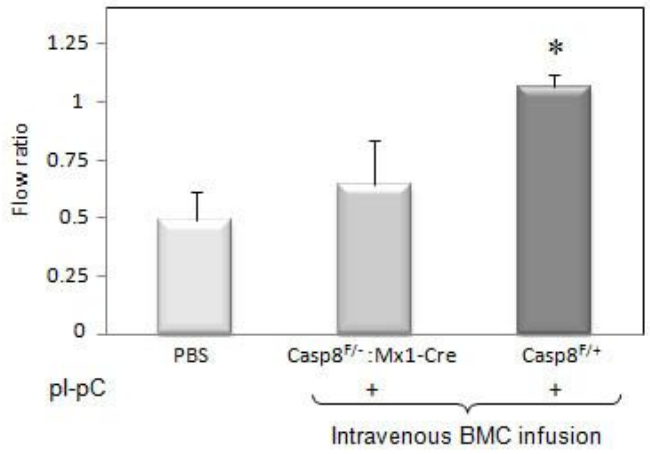


Figure 3.7.4: Post-ischemic neovascularization of bone marrow mononuclear cells from mice with a conditional caspase-8 deletion

Bone marrow mononuclear cells with a caspase-8 deletion have a reduced neovascularization capacity compared to cells with two functional caspase-8 alleles. One million cells were intravenously infused. Two weeks later, blood flow of hind limbs was

measured with a laser Doppler blood flow meter. The blood flow ratio based on the comparison of ischemic to non-ischemic hind limb perfusion. No cells were infused in the PBS group. (* $p<0.05$ vs. PBS), n=4 animals per group.

Moreover, capillary density from hind limb muscles was analyzed by staining frozen sections with antibodies against $CD31^+$ and laminin to visualize small new capillaries around existing myocytes. Caspase-8-deficiency reduced the functional capability of bone marrow mononuclear cells to increase capillary density after hind limb ischemia (Fig.3.7.5), and supported our idea that caspase-8 is indeed required for precursor cell-mediated functional improvement of neovascularization *in vivo*.

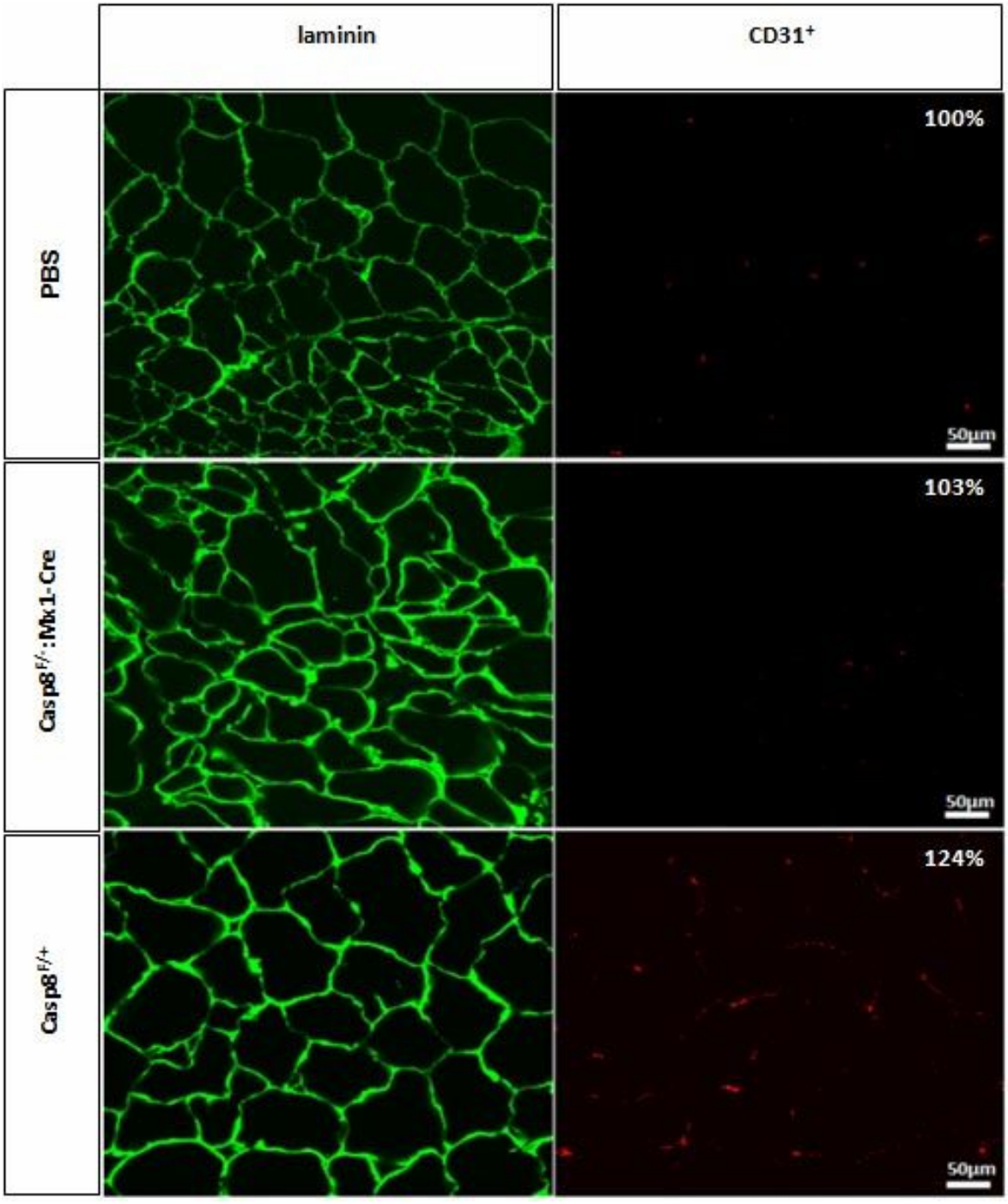


Figure 3.7.5: Fluorescence images of capillary density from hind limb of nude mice

Stainings of frozen sections from the right hind limb following hind limb ischemia and infusion of bone marrow cells from mice with induced caspase-8 deletion or control mice without Cre (PBS (100%) vs. $Casp8^{F/-}$:Mx1-Cre(103%) vs.$Casp8^{F/+}$(124%)). Muscles were harvested two

weeks after ischemia. Numbers enclosed in brackets indicate the ratios of capillaries to myocytes (ratio of PBS group was set to 100%).

Surface receptors like integrins and CXCR4 are important for homing, vascularization and angiogenesis (Chavakis, Aicher et al. 2005) (Yamaguchi, Kusano et al. 2003) (Walter, Haendeler et al. 2005). FACS analysis revealed that bone marrow mononuclear cells from induced caspase-8-deficient mice express significantly lower levels of integrin alpha5, CXCR4 and integrin alpha4. *In vivo*, the expression of VEGF-R2 (KDR) did not change significantly after Cre recombinase induction by interferon γ (Fig.3.7.6a-d).

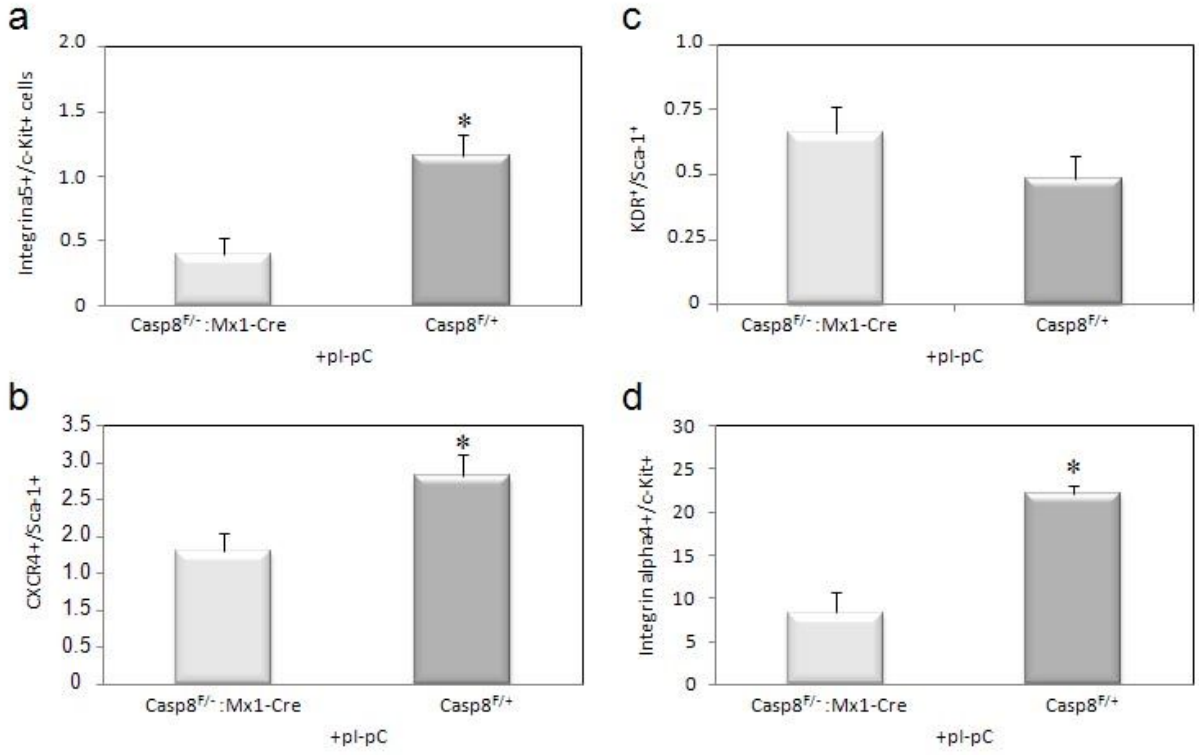


Figure 3.7.6: FACS analysis of mice BMC

Double-staining of mice BMCs with antibodies against integrin alpha5, CXCR4, VEGFR2 and integrin alpha4 combined with c-Kit or Sca-1 (markers for hematopoietic progenitor stem cells). Comparison of BMCs of control mice ($Casp8^{Flox/+}$) with cells of mice with caspase-8 deletion ($Casp8^{F/-}$:Mx1-Cre) after Cre recombinase induction (* $p<0.05$ vs. $Casp8^{F/-}$:Mx1-Cre), n=3 animals per group.

3.8 Cell surface receptor regulation by caspase-8 inhibition

Because of the reduced cell surface expression of integrin alpha5, CXCR4 and integrin alpha4 in caspase-8 deficient BMCs, we investigated possible downstream effectors influencing surface receptors in EPCs.

We performed FACS analysis of EPCs following caspase-8 inhibition by zIETD. We tested the EPCs for different adhesion and homing molecules. As already shown treatment with 100 µM zIETD induced irreversible detachment of the cells (Fig.3.3.1). FACS analysis showed that the fibronectin receptor subunits integrin alpha5 and to a lower extent integrin beta1 are reduced on the cell surface after caspase-8 inhibition (Fig.3.8.1a). Furthermore, the vitronectin receptor subunits αVβ5 and αVβ3 are significantly reduced on the EPC surface, but not as strong as the integrin subunit alpha5 (Fig.3.8.1b). Additional analysis of CD18 and CD11a,b,c (Fig.3.8.1c) showed only significant reduction in CD11a after 3 h of treatment with zIETD and zVAD, a pan inhibitior for all caspases.

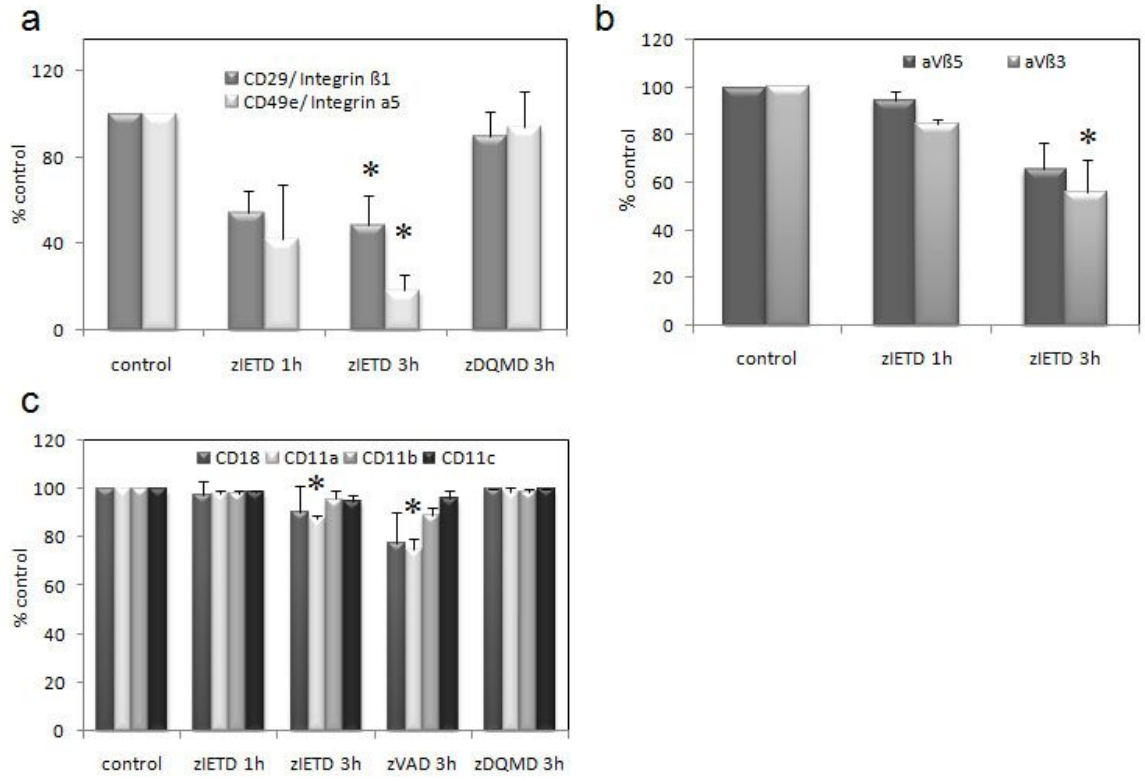


Figure 3.8.1: FACS analysis of EPCs

(a-c) Analysis of different adhesion molecules of the integrin family on the cell surface of EPCs by fluorescence activated cell sorting followed by caspase inhibition (100 µM), * $p<0.05$ vs. control, each n=3.

Consistent with the oberservation that inhibition of caspase-8 impairs SDF1α stimulated migration (Fig.3.3.2a), pharmacological caspase-8 inhibition targets C-X-C chemokine receptor type (CXCR4) surface expression on EPCs. There is a significant reduction in CXCR4 on EPC (Fig.3.8.2a). Similar but less pronounced results were obtained for VEGF-R2 expression (Fig.3.8.2b).

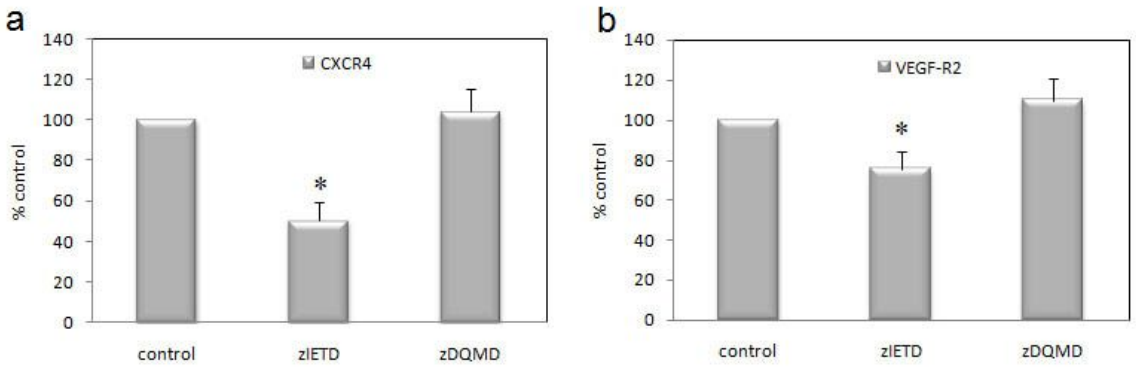


Figure 3.8.2: CXCR4 and VEGF-R2 surface expression is dependent on caspase-8

FACS analysis of EPCs after 4 hours of caspase-8 (zIETD) or caspase-3 and -6 (zDQMD) inhibition (100 µM), * p<0.05 vs. DQMD, n=4.

Because of the strong reduction of integrin alpha5 on the cell surface analyzed by FACS, we checked for the protein expression of the fibronectin subunits alpha5 and beta1. Western blot analysis showed a reduction in protein levels of integrin alpha5 following caspase-8 inhibition by zIETD (Fig.3.8.2a) but not of beta1 expression (Fig.3.8.2b). Protein expression was compared with cell extract derived from EPCs pretreated with zDQMD (inhibits caspase-3 and -6).

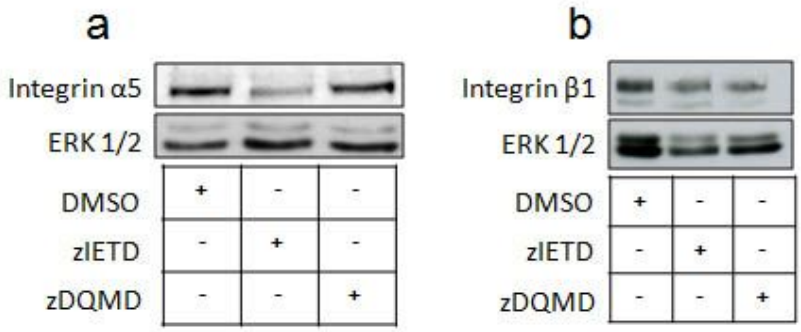


Figure 3.8.2: Western blot from EPC

(a, b) Integrin alpha5 and beta1 protein detection following ex vivo treatment of endothelial precursor cells with caspase inhibitors (100 µM) for 4 hours. Caspase-8 inhibition by zIETD significantly reduced integrin alpha5 protein expression compared to control (solvent) and zDQMD but not beta1 protein expression. (a) n=5, (b) n=4

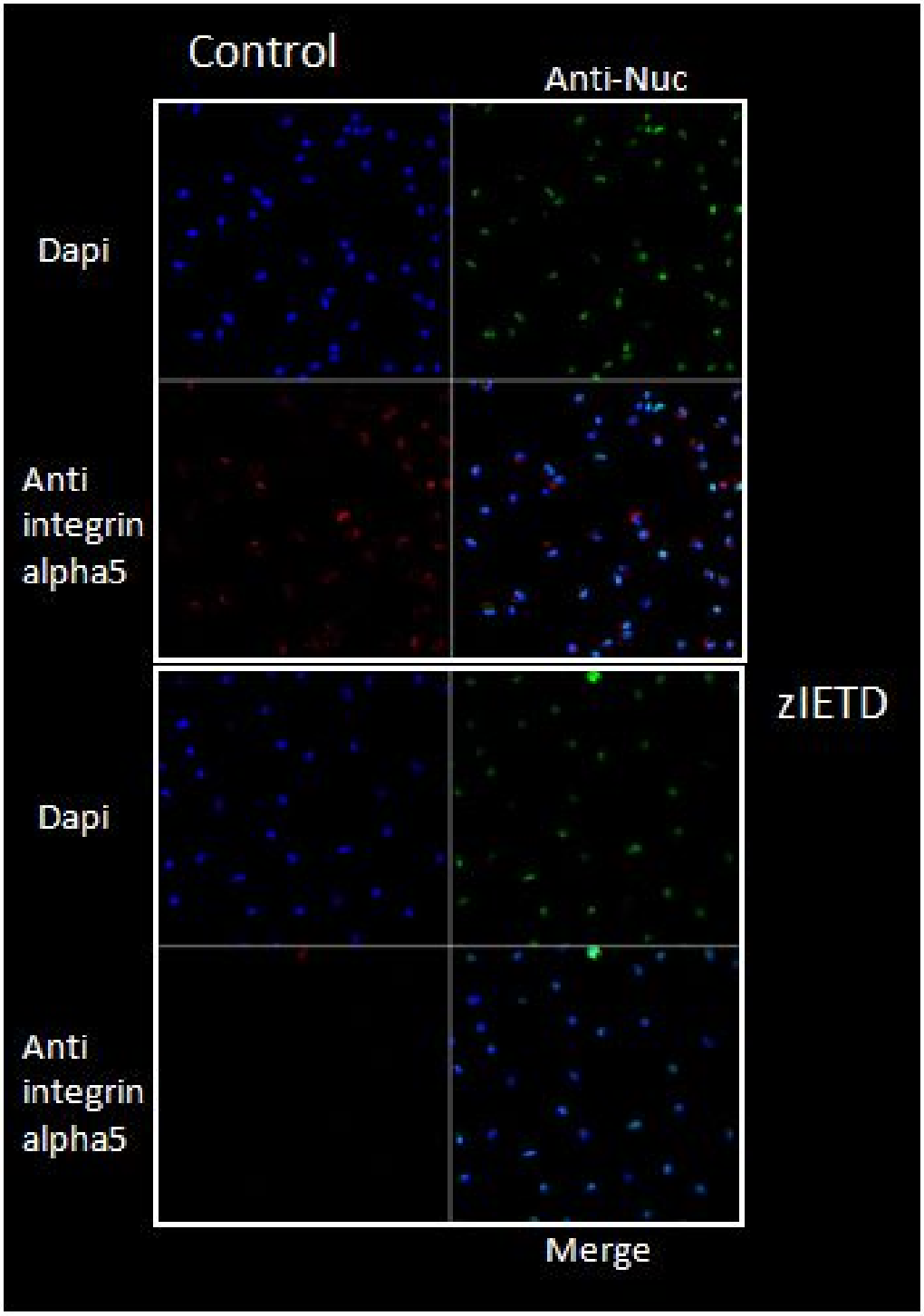


Figure 3.8.3: Integrin alpha5 expression after zIETD short-time treatment.

Representative confocal microscopy images of paraformaldehyd–fixed EPCs after caspase-8 inhibition by zIETD (100 µM) for 30 min and staining with antibody against integrin alpha5, DNA-fluorescence dye DAPI and anti-nucleosom (anti-Nuc).

EPCs, which were treated with zIETD for 30 minutes, were fixed and stained for integrin alpha5, DAPI and anti-nucleosom. Already short-time incubation with zIETD reduced the expression level of integrin alpha5 in EPCs compared to untreated cells (Fig.3.8.3).

To determine whether the reduction of the alpha5 subunit is sufficient to explain the reduced adhesion shown after zIETD treatment, we co-incubated EPCs with a neutralizing integrin alpha5 antibody. As shown in Figure 3.8.4, indeed, blocking the integrin alpha5 subunit significantly reduced the adhesion of EPCs to the matrix protein fibronectin.

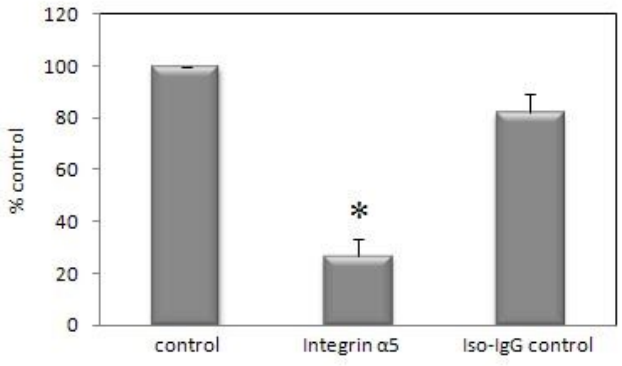


Figure 3.8.4: EPC adhesion on fibronectin

Cell adhesion to fibronectin was significantly reduced following incubation with a neutralizing antibody against integrin α5 for 6 hours, * $p<0.005$ vs. iso-IgG antibody as control; n=4

3.9 Caspase-8 targets Cbl-b

Many caspase substrates have been identified in the past (Kang, Ben-Moshe et al. 2004) (Frisch 2008) (Maelfait and Beyaert 2008). Potential candidates to modulate the migration and adhesion capacities of EPCs are the Cbl E3 ubiquitin ligases, which were identified to be degraded in a caspase-8-dependent manner (Widmann, Gibson et al. 1998). Cbl proteins act as negative regulators of various signal transduction proteins involved in cell migration and attachment (reviewed in (Schmidt

and Dikic 2005)). Expression profiling revealed that among the Cbl proteins the isoform Cbl-b is particularly highly expressed in EPC (Fig.3.9.1).

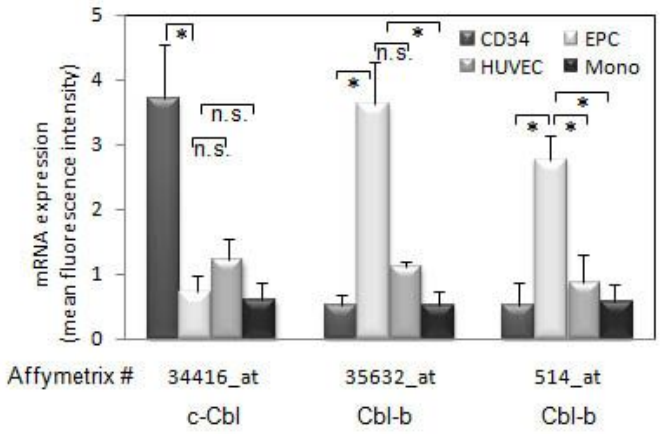


Figure 3.9.1: Cbl expression in different cell types

In a microarray, the mRNA expression level of Cbl protein was detected. The Cbl-b isoform was significantly higher in EPC compared to $CD34^+$, monocytes (Mono) and HUVEC. c-Cbl isoform was low expressed in EPC similar to HUVEC and monocytes. n=3, * $p<0.05$ (mean ± SEM)

In order to test, whether Cbl-b is a target of caspase-8, we overexpressed Cbl-b wildtype in HEK293 cells.

As shown before, caspase-8 inhibition leads to integrin alpha5 reduction (Fig.3.8.2). Further, caspase-8 inhibition by the pharmacological substrate zIETD increased Cbl-b protein level in EPC (Fig.3.9.2). These results could be a first hind for signal transduction from caspase-8 to Cbl-b and this in turn leading to the downregulation of integrin alpha5.

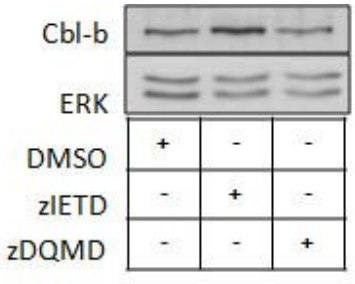


Figure 3.9.2: Protein analysis of stimulated EPC

EPCs were treated with caspase inhibitors (100 µM, 4 h), and protein was detected by Western blot. Cbl-b protein was upregulated in the presence of caspase-8 inhibitor compared to control and zDQMD. ERK1/2 was used as loading control. n=6

Because of the inefficient lentiviral transduction in EPCs (Fig.3.7.1), further experiments were performed in HEK293 cells. In HEK293 cells, endogenous as well as overexpressed wild type Cbl-b was degraded in the presence of simultaneously overexpressed caspase-8 (Fig.3.9.3).

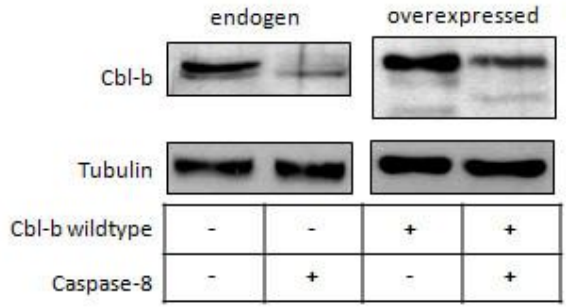


Figure 3.9.3: Simultanous overexpression of Cbl-b and caspase-8 in HEK293T cells

Endogenous and overexpressed protein of Cbl-b in HEK293 cells was degraded in the presence of simultanously overexpressed caspase-8. Antibody against Tubulin was used as loading control. n=3

These results support the hypothesis that caspase-8 targets Cbl-b in EPCs. Additionally, overexpression of Cbl-b in HEK293 cells and subsequent incubation of the cell lysate with recombinant caspase-8 protein showed a decrease in Cbl-b protein amount (Fig.3.9.4).

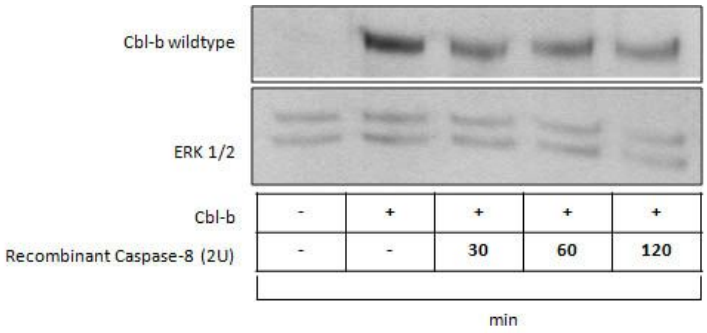


Figure 3.9.4: Cell-free cleavage of overexpressed Cbl-b

Overexpression of Cbl-b wildtype protein and incubation of the cell lysate with recombinant caspase-8 (2 Units) at 37°C for the indicated periods of time showed a decrease in the overexpressed protein, n=3.

Afterwards, immunoprecipitation of overexpressed Cbl-b followed by incubation with recombinant caspase-8 revealed an effective degradation of Cbl-b protein (Fig.3.9.5). These results suggest that caspase-8 targets Cbl-b and thereby, indirectly influences integrin alpha5 protein expression.

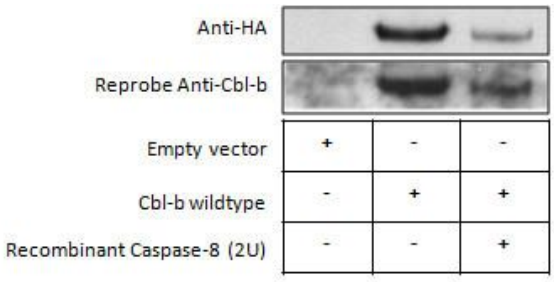


Figure 3.9.5: Degradation of Cbl-b protein by caspase-8

Overexpression of Cbl-b in HEK293 cells was followed by immunoprecipitation against HA-Tag with specific antibody (Roche®). *In vitro* degradation of immunoprecipitated Cbl-b protein was performed using recombinant Caspase-8 for 30 min, n=3.

Using different truncated Cbl-b constructs, we aimed to identify the cleavage site of caspase-8 inside Cbl-b (Fig.3.9.6). These constructs have overlapping regions.

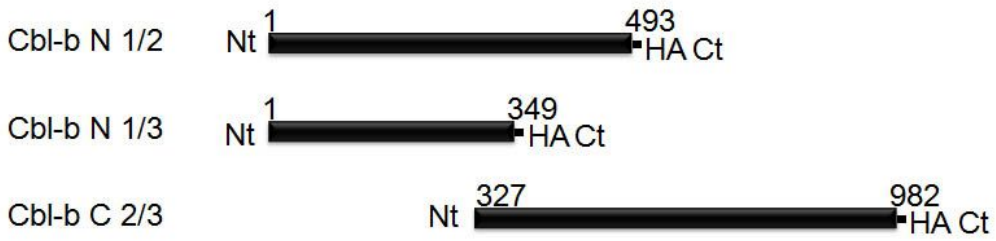


Figure 3.9.6: Truncated Cbl-b constructs

Schematic presentation of different truncated Cbl-b plasmid constructs based on the Cbl-b wildtype (kindly provided by I.Dikic group, Institute of Biochemistry, Frankfurt).

Truncated Cbl-b constructs (which are kindly provided by I.Dikic) were strongly overexpressed with exception of Cbl-b N1/3. In this case, overexpression appeared to be very low. But caspase-8 cleaved all Cbl-b constructs (Fig.3.9.7) resulting from more than one cleavage site. Cleaved fragments are not detected assuming a fast degradation or instability of the dysfunctional elements.

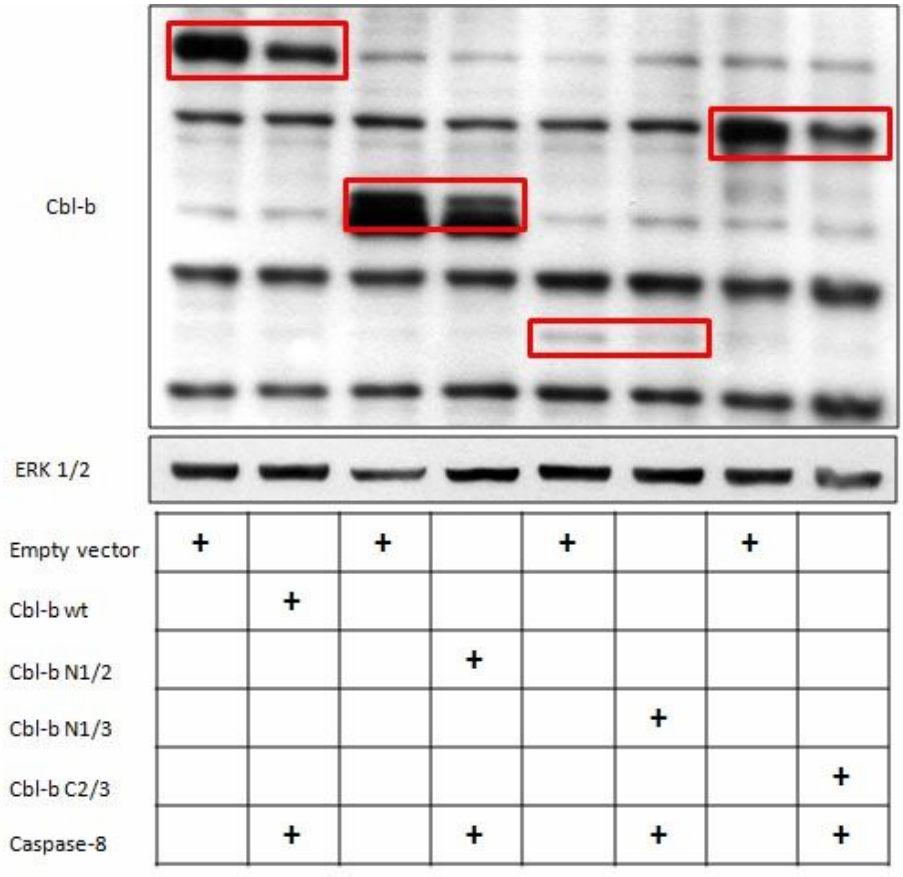


Figure 3.9.7: Simultanous overexpression of truncated Cbl-b constructs with caspase-8 wildtype

Simultanous overexpression of HA-epitope tagged Cbl-b fusion proteins and caspase-8 wildtype in HEK293 cells with Superfect reagent. Detection of the Cbl-b constructs with antibody against HA-Tag, n=3.

These data strongly indicates that caspase-8 regulates indirectly the fibronectin subunit integrin alpha5 in EPC via the E3 ubiquitin-protein ligase Cbl-b.

3.10 Cbl-b influences angiogenesis

Because of the limited transfection efficiency in EPCs, in vitro downregulation of Cbl-b was investigated in HUVEC. A specific siRNA targeting Cbl-b was used to down

regulate Cbl-b. Down regulation was confirmed by semiquantitative RT-PCR (Fig.3.10.1).

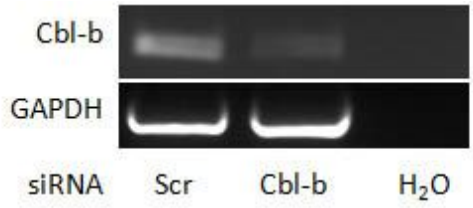


Figure 3.10.1: Expression and knockdown of Cbl-b

HUVEC were transfected with siRNA targeting Cbl-b using Superfect reagent. mRNA expression level was detected by RT-PCR 24 hours after transfection. GAPDH served as loading control.

After siRNA transfection, HUVECs were subjected to a three-dimensional angiogenic spheroid assay to analyze sprouting capacity *in vitro*. Indeed, Cbl-b downregulation leads to an increase in endothelial cell sprouting under basal conditions and suggest that Cbl-b has a critical role in angiogenic activity (Fig.3.10.2).

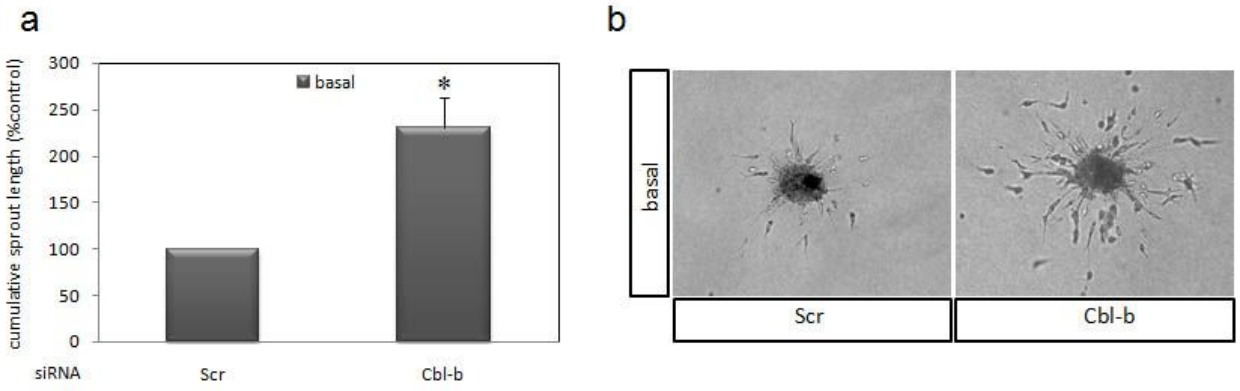


Figure 3.10.2: Spheroid assay of HUVEC after downregulation of Cbl-b

(a) HUVECs were transfected with Cbl-b siRNA or scrambled oligonucleotides. Spheroid assays were performed to analyze basal endothelial cell sprouting capacity. Sprouting capacity is given as cumulative sprout length per spheroid, * $p<0.05$ vs. scramble (Scr) (mean±SEM), n=3. (b) Representative spheroids are shown for scrambled and Cbl-b siRNA.

To assess if the observed pro-angiogenic effect *in vitro* is based on enhanced migration, we used Cbl-b siRNA treated HUVECs in the boyden chamber migration assay to address this aspect. Cbl-b knockdown in HUVEC significantly increased migration. These results are consistent with the angiogenic skills of Cbl-b-siRNA-transfected HUVECs in the spheroid assay (Fig.3.10.3).

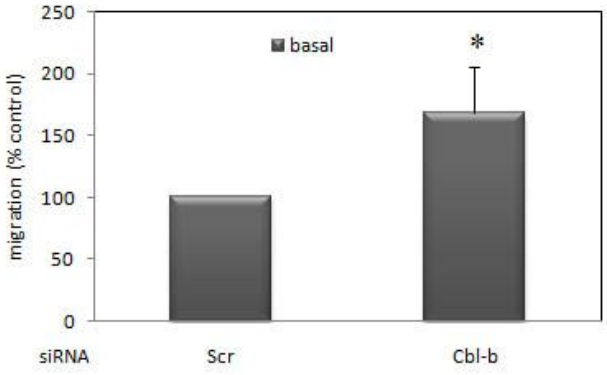


Figure 3.10.3: HUVEC migration after Cbl-b siRNA knockdown

HUVECs were transfected with siRNA oligonucleotides against Cbl-b and scrambled sequence (Scr). Migration was performed in fibronectin coated boyden chambers for 5 h. After fixation of the cells, DAPI staining was used to count migrated cells. * $p<0.05$ vs. scramble (mean±SEM), n=3.

3.11 Cbl-b in vascularization in vivo

Having demonstrated that caspase-8 targets Cbl-b in EPCs, we next tested whether Cbl-b modulates angiogenesis *in vivo*. For this purpose, a Cbl-b knockout mouse model was used to investigate involvement of Cbl-b in vascularization and angiogenesis (Bachmaier, Krawczyk et al. 2000).

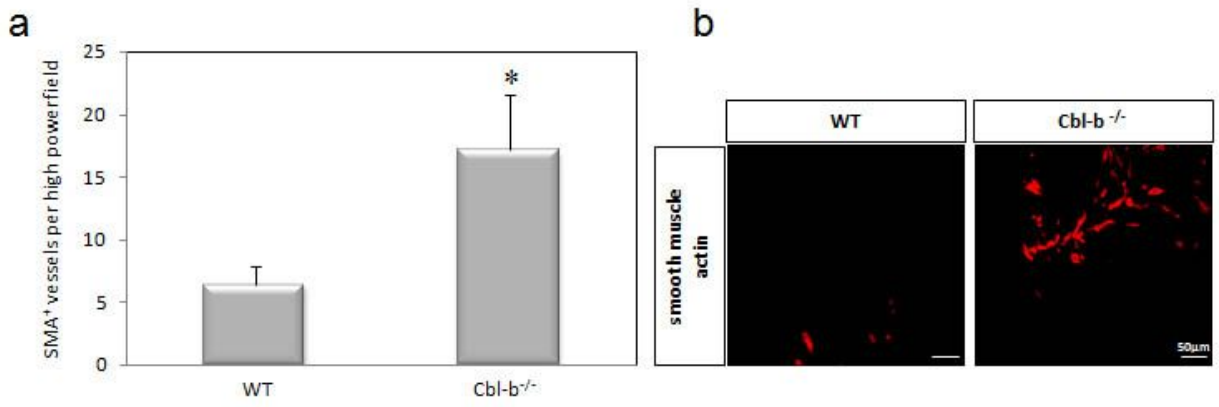


Figure 3.11.1: Involvement of Cbl-b in angiogenesis *in vivo*

Vascularization of matrigel plugs in Cbl-b-deficient mice were compared to wild type animals, measured by quantification of smooth muscle actin positive vessels, * $p<0.05$ (mean±SEM), each group n=7.

Vascularization was detected and quantified in paraffin-embedded matrigel plugs by staining for smooth muscle actin. Indeed, vascularization of matrigel plugs implanted in the back of Cbl-b deficient mice was strongly increased compared to wild type mice (Fig.3.11.1a/b).

In a hind limb ischemia model, post-ischemic perfusion was more effectively rescued by transplanted bone marrow progenitor cells derived from Cbl-b-deficient mice (Fig.3.11.2) compared to BMCs from wild type mice. Thus, Cbl-b could act as a negative regulator of vasculogenic precursor cell function and angiogenesis *in vivo*.

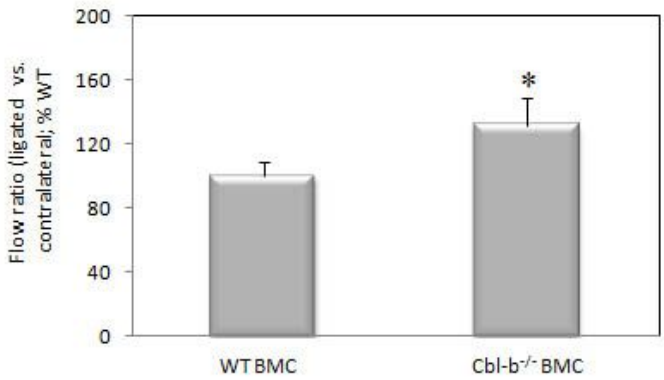


Figure 3.11.2: Post-ischemic neovascularization of transplanted bone marrow mononuclear cells from Cbl-b deficient mice

After two weeks, neovascularization in femoral artery ligated nude mice was enhanced after the transplantation of bone marrow mononuclear cells derived from Cbl-b-deficient (Cbl-b$^{-/-}$) mice compared to wild type BMCs, measured by Laser Doppler. Flow rate is given as ratio of the ligated vs. contralateral limb; * $p<0.05$ (mean±SEM), n=6 per group.

3.12 Regulation of Caspase-8

Having demonstrated that EPCs reduce the release of cytokines like chemokine stromal cell-derived factor (SDF) -1 and hepatocyte growth factor (HGF) (3.4.) under pharmacological caspase-8 inhibition, we questioned how caspase-8 is further regulated besides the known apoptosis induction by death receptors like TNF or Fas? One possible opportunity could be the influence of cytokines as well as mediators from the inflammatory tissue.

In a three day culture assay, Interleukin-6 significantly increased the fibronectin-adherent endothelial precursor cells (Fig.3.12.1) as measured by counting cells taking up Ac-Dil-LDL.

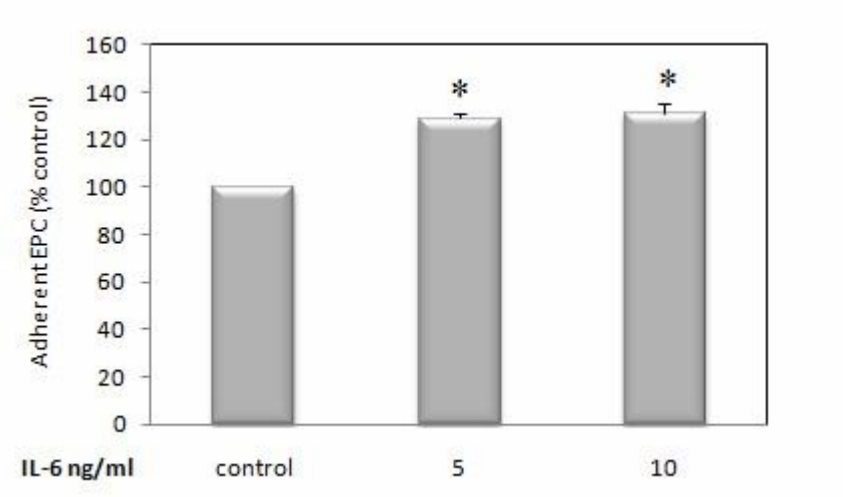


Figure 3.12.1: Effect of Interleukin-6 on EPC formation

Mononuclear cells were isolated from blood and cultured for 72 hours in the presence of Interleukin-6 (5 or 10ng/ml). The number of adherent EPCs increased in combination with

interleukin-6 stimulation. Formation of adherent precursor cells was counted with a microscope after staining the cells with Dil-Ac-LDL and vWF, * p<0.05 (mean±SEM), n=5.

Furthermore, Western blot analysis of EPC lysates showed that IL-6 stimulation decreased Cbl-b protein amount (Fig.3.12.2).

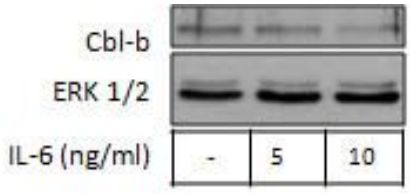


Figure 3.12.2: Protein expression of Cbl-b after Interleukin-6 stimulation

Mononuclear cells were isolated and cultured *ex vivo* 72 hours in combination with Interleukin-6. Cbl-b protein was detected with a specific antibody (Santa Cruz). The expression of Cbl-b was decreased with an increased stimulation of interleukin-6. n=3.

Furthermore, FACS analysis of integrin alpha5 in day 3 EPCs confirmed this possible caspase-8 regulation and matched to the previous data. Integrin alpha5 was significantly increased in EPCs treated with Interleukin-6 (Fig.3.12.3).

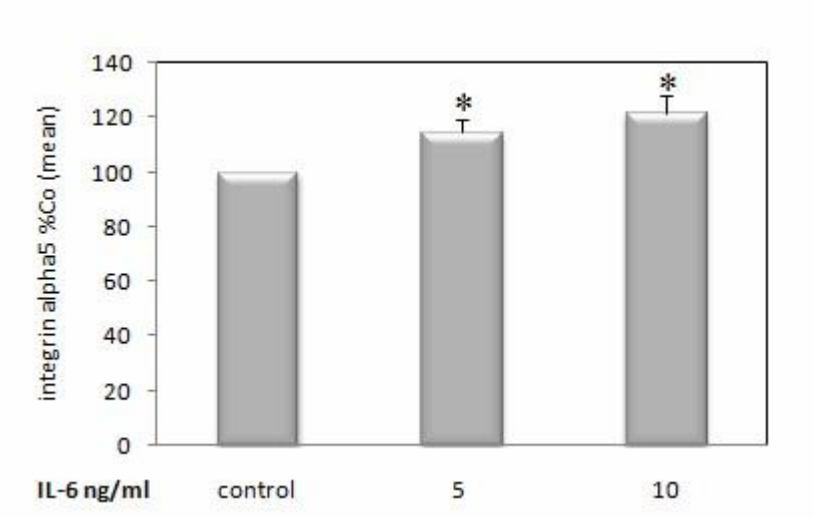


Figure 3.12.3: Integrin alpha5 expression on EPC after Interleukin-6 stimulation

Integrin alpha 5 surface expression on EPCs was significantly increased after interleukin-6 (5 and 10 ng/ml) stimulation measured by flow cytometry, * p<0.05 vs. control (mean±SEM), n=8.

4. Discussion

Endothelial progenitor cells comprise a therapeutic neovascularization-promoting potential after ischemia which is due to survival and retention of the infused cells in the ischemic tissue. Caspases are mainly described as mediators in the apoptotic pathway induction. However, detailed investigations in the past decade revealed that caspases are additionally involved in other biological processes like migration, proliferation and differentiation (reviewed in (Launay, Hermine et al. 2005) (Lamkanfi, Festjens et al. 2007)). Since caspase function in endothelial precursor cells is mainly unknown so far, this study is basically focussed on the function of Caspases and their non-apoptotic targets in EPC regulation.

4.1 Characterization of endothelial progenitor cells

Endothelial precursor cells are a heterogeneous cell population, mostly isolated from peripheral (Asahara, Murohara et al. 1997) or umbilical cord blood (Murohara, Ikeda et al. 2000) and bone marrow (Lin, Weisdorf et al. 2000). The mononuclear cells isolated from peripheral blood by ficoll density gradient centrifugation and cultured in endothelial differentiation conditions express endothelial, myeloid and hematopoietic markers (Fig.3.1.1). The expression of endothelial marker protein VEGF-R2, von Willebrand factor (vWF) and lectin binding was shown by FACS analysis, whereas endothelial nitric oxide synthase (eNOS) was previously described by our group (Dernbach, Urbich et al. 2004) (Chavakis, Aicher et al. 2005) as well as uptake of Ac-LDL. However, since EPC does not express cell specific markers, they are still difficult to characterize. EPC are divided into early and late stage subpopulations because of variations in expression levels of different markers (Hur, Yoon et al. 2004). After prolonged cultivation, cells maturate and simultaneously endothelial marker genes further increase. Currently, isolated and cultured cells used in our studies might be characterized as early EPC because of parallel expression of hematopoietic and endothelial markers. They express the neutrophil and macrophage marker CD31 and vascular endothelial (VE)-cadherin (Dimmeler, Aicher

et al. 2001) as well as the hematopoietic leukocyte marker CD45 (Urbich, Heeschen et al. 2003) and stem cell homing receptor CXCR4.

Variations in isolation and culture conditions of EPC have been shown to influence the subpopulation as well as the differentiation status and subsequently the expression of protein markers (Eggermann, Kliche et al. 2003) (Seeger, Tonn et al. 2007).

4.2 Selection of Caspase-8 as an important player in EPC function

Caspases are pro-apoptotic enzymes but investigations allocate them non-apoptotic roles in many species and cell types (Lamkanfi, Festjens et al. 2007). Pharmacological inhibitors are commercially available to competitively and irreversibly inhibit caspases by mimicking substrates. At the beginning of this study we planned to use caspase inhibition to improve incorporation and survival of therapeutically applied EPC in ischemic tissue. Sordet et al. showed that several short term active caspases in peripheral blood monocytes are involved in the differentiation into macrophages, and this process was blocked by caspase inhibitors (Sordet, Rebe et al. 2002). We also could show in first experiments that caspases have other tasks in EPC besides apoptosis induction. Surprisingly, in our hands the pan caspase inhibitor as well as the specific caspase-8 inhibitor zIETD completely abrogated the adherence of EPC on fibronectin *in vitro* (Fig.3.1.2 a,b). Additionally, in our hands *ex vivo* zVAD treated EPC lost their ability in adhesion and migration. These findings assumed a novel role of Caspase-8 in EPC formation and EPC retention in injured tissue and could be helpful for better understanding the progenitor cell-mediated neovascularization. Moreover previous observations demonstrate already an essential function of caspase-8 in endothelial cells during embryonic vascular development and hemopoiesis (Kang, Ben-Moshe et al. 2004).

4.3 Low Caspase-8 expression in HUVEC

Having shown that treatment of EPC with the pan caspase inhibitor zVAD as well as the specific Caspase-8 inhibitor zIETD resulted in inhibition of formation and adhesion of EPC, we aimed to analyze the caspase-8 functions in peripheral blood mononuclear cell-derived EPC in more detail. Caspases are synthesized as inactive proenzymes containing 3 domains: a N-terminal domain, a large subunit and a small subunit. Recruitment of caspase-8 by adaptor proteins like FADD (Fas-associated protein with death domain) leads to its auto-activation followed by a heterodimer formation (Thornberry and Lazebnik 1998). The expression of caspase-8 itself is regulated by alternative splicing. In the literature, several isoforms are described for caspase-8 (Boldin, Goncharov et al. 1996). Caspase-8 a/b comprise the complete forms which are known to mediate apoptosis. One alternative splice variant of caspase-8 is caspase-8L (Horiuchi, Himeji et al. 2000). Caspase-8L transcript carries a 136 bp insertion finishing in a premature termination by a stop codon. The protein carries two repeats of DED of full-length caspase-8 N-terminally but lacks the C-terminal proteolytically active domain. Caspase-8L is described as endogenous inhibitor of caspase-8 by binding to FADD (Fas-associating protein with death domain) and caspase-8 and interferes the binding between them (Himeji, Horiuchi et al. 2002). Expression of caspase-8L was found in many tissues and peripheral blood lymphocytes (Himeji, Horiuchi et al. 2002) and prevents $CD34^+$ hematopoietic progenitor cells from CD95 receptor-mediated apoptosis (Mohr, Zwacka et al. 2005).In a first approach, we analyzed caspase-8 expression in EPC on mRNA and protein level by western blot analysis and confocal imaging. Additionally, we compared Caspase-8 mRNA levels in different cell types (Fig.3.2.1a,b,c). Our analysis by RT-PCR revealed that caspase-8L is expressed only in freshly isolated mononuclear cells compared to very low level in day 3 suspension cells and adherent day 3 EPC. In contrast, HUVEC did not express caspase-8L (Fi.3.2.1.a lower panel). These results let assume that caspase-8L as an inhibitor of apoptosis induction is only expressed in hematopoietic cells. Perhaps, transcription of this splice variant is repressed during cell differentiation. Furthermore, caspase-8 is generally less

transcribed in HUVEC compared to monocytes, $CD34^+$ and EPC (Fig.3.2.1c). Caspase-8 as an initiator of apoptosis assumes a localization in the cytoplasm triggered by outside-in-signaling close to the cell membrane. However, we detected caspase-8 in the cytoplasm as well as in the nucleus by confocal images (Fig.3.2.1b). This observation was already described by Besnault-Mascard et al. They found nuclear localization of caspase-8 associated with sumoylation in the N-terminal DEDs. The localization of the nuclear p75 (75kDa) caspase-8 was controlled by a SUMO-1-dependent pathway, whereas non-sumoylated p50-55 caspase-8 forms are present in the cytoplasm (Besnault-Mascard, Leprince et al. 2005). In our study, the non-sumoylated p50-55 active form of caspase-8 in the cytoplasm is part of our investigations where direct and close interaction to cytoplasmic signaling proteins are important.

4.4 Non-apoptotic function of Caspase-8

Caspase-8 as an initiator of programmed cell death induction activates executioner caspase-3 by cleavage (Cohen 1997), which cleaves other downstream targets to switch on the apoptosis machinery. Measurements of caspase-8 and -3 activity showed that high caspase-8 activity in EPC compared to peripheral blood mononuclear cells and to adherent cells at day 3 of culture did not increase caspase-3 activity (Fig.3.5.1a,b). In this case, activity of caspase-8 does not induce apoptosis as shown by measuring apoptotic cells with Annexin V (Fig.3.5.2). In accordance, other results indicates that caspase-3 is, beside apoptotic signaling, also involved in other cellular processes like embryonic stem cell differentiation (Fujita, Crane et al. 2008) and alters the sensitivity to cytokine stimulation in hematopoietic stem cells (Janzen, Fleming et al. 2008). However, mice with genetic gene deletion of caspase-3 are born alive without vascular defects (Woo, Hakem et al. 2003). Dernbach et al. revealed that EPC are more resistant to oxidative stress and less sensitive to ROS (reactive oxygen species)-induced apoptosis compared to other cell types. They express higher portions of catalase, glutathione-peroxidase and manganese superoxide dismutase than mature endothelial cells (Dernbach, Urbich et al. 2004).

Higher levels of caspase-8 and subsequent apoptosis induction might be blocked by the antioxidative possibilities of EPC. Additionally, some metastatic tumor cells express active caspase-8 which generates programmed cell death sensitivity but is also able to suppress metastasis (Helfer, Boswell et al. 2006). Caspase-8 inhibition in EPC could influence a similar process like in metastatic tumor cells by disturbing integrin-mediated attachment signaling. Concluding from our caspase-3 results, caspase-8 may act directly rather than through caspase-3 to cleave substrates critical for cell motility although other effector caspases cannot be excluded (Helfer, Boswell et al. 2006).

4.5 Cell adhesion and migration in the presence of caspase-8 inhibitor is due to transmembrane molecule reduction

Recent reports described a role of caspases in the control of sperm individualization, cell morphology and migration (Lamkanfi, Festjens et al. 2007) (Li, Brieher et al. 2007). Furthermore, adhesion and migration ability of different cells are important features for vascularization in new growing vessels. Since caspase-8 inhibition blocked the formation of fibronectin-adherent EPC, we questioned weather EPC adhesion in response to caspase-8 is specific for fibronectin. Dysregulation of integrin α5 is known to be essential for embryonic vessel formation as demonstrated by vascular defects in integrin α5-deficient mice (Yang, Rayburn et al. 1993). In order to solve this question we performed adhesion assay using different matrices such as recombinant fibronectin, ICAM-1 and collagen I. Adhesion to all matrices was completely abolished, when the EPC were pretreated with caspase-8 inhibitor zIETD and then replated (Fig.3.3.1). This observation excluded the specificity of caspase-8 inhibition to α5β1-integrin, the specific receptor of fibronectin. The adhesion to ICAM-1 via ß2-integrins was blocked to a similar extent. Since the observed effect was not specific for fibronectin, we could conclude that adhesion is not only dependent on integrin α5. In contrast to our results, pharmacological inhibition of caspase-8 in breast epithelial cells (MFC10A) did not influence adhesion (Finlay and Vuori 2007) demonstrating specific function of caspase-8 in EPCs. Additionally, in our hands

migration was blocked by caspase-8 inhibitor in EPCs independent of application of the chemokines SDF-1, VEGF and IL-8, which are known to stimulate migration by attracting cells (Fig.3.3.2a,b,c). These findings might be well explained by the reduced surface expression of various integrins like α5, β1, αvβ3, CD11a (3.8.1a-c) and the chemokine receptor CXCR4 (3.8.2a). The integrin subunit α5, however, was most efficiently downregulated by treatment of EPCs with zIETD (Fig.3.8.1a). Furthermore, we already know that ß2-integrins are important for EPC adhesion and transmigration (Chavakis, Aicher et al. 2005). Moreover, integrin α5 was reduced in whole cell protein lysates, FACS analysis and fluorescence staining. In contrast, β1 and β2 integrins were not regulated on protein level. Since regulation of β1 and β2 integrins on protein level are missing, caspase-8 inhibition might additionally block adhesion by interfering with intracellular signaling. CXCR4 is a transmembrane receptor expressed by peripheral blood lymphocytes, monocytes, thymocytes, dendritic cells and endothelial cells which is critical in embryonic vascularization. Caspase-8 inhibition by zIETD significantly reduced the chemokine receptor CXCR4 (3.8.2a) and lead to the ineffective attraction by its ligand SDF-1 (3.3.2a). The CXCR4 receptor is important for the proangiogenic potential of EPCs (Zemani, Silvestre et al. 2008). In addition, the expression of the endothelial marker protein VEGF-receptor 2 (KDR) was slightly decreased by about 20% after addition of the caspase-8 inhibitor. Interestingly, HUVEC and HMVEC migration was not affected by different caspase inhibitiors. This lack of response might be explained by the low caspase-8 mRNA expression in these mature endothelial cells. The results indicate that caspase-8 activity controls integrins and chemokine-receptors involved in matrix adhesion and migration of EPC. Unfortunately, since we were not successful in transducing EPC with lentiviral vectors provided, we were unable to perform rescue experiments by overexpression of integrin α5, β1 and CXCR4 simultanously to the inhibition of caspase-8. In summary we demonstrated that inhibition of Caspase-8 impairs migration and adhesion of circulating blood or bone marrow-derived hematopoietic cells. Because of the low Caspase-8 expression, mature endothelial cells are not affected by Caspase-8 inhibitors. However, caspase-8 is not the only caspase involved in the adhesion and migration machinery. Caspase-11 has been

also shown to be involved in cytoskeleton organization by mediating depolymerization in lymphocytes (Li, Brieher et al. 2007).

4.6 Growth factor production is influenced indirectly by caspase-8

Since EPC derive from hematopoietic precursors, one may argue that inhibition of EPC proliferation or differentiation or alternatively an influence on cytokine expression caused the abrogation of adherent EPC by caspase-8 inhibition. Indeed, caspase-8 inhibitor treatment modulated the release of cytokines in vitro. Thus, the release of SDF-1 and HGF was significantly reduced whereas IGF-1 was slightly but not significantly increased. However, the profound and rapid effects of the caspase-8 inhibitor on cell adhesion and on α5β1-expression are unlikely caused by an inhibition of differentiation or modulation of cytokine levels. Most likely, the effects on differentiation and on cytokine levels are secondary to the impaired interaction of α5β1-integrin to fibronectin and the reduced number of cytokine-producing adherent cells after caspase-8 inhibition.

4.7 Caspase-8 inhibition in EPC impedes neovascularization

Tissue neovascularization in hind limb ischemia or myocardial infarction is enhanced by transplanted endothelial precursor cells (Kalka, Masuda et al. 2000) (Kawamoto, Gwon et al. 2001). In accordance to previous results from our group and other investigators, the transplantation of EPC into athymic nude mice increased blood flow recovery and prevented limb necrosis by incorporation of EPC into the ischemic area (Urbich and Dimmeler 2004). By contrast, pretreatment of EPC with pharmacological caspase-8 inhibitors reduced incorporation of cells in the ischemic tissue as well as perfusion and blood flow recovery of the ischemic limb (Fig. 3.6.1a,b and 3.6.2). In order to analyze caspase-8 deficiency without the use of pharmacological inhibitors, we used bone marrow cells from conditional caspase-8 knockout mice induced by the interferon-responsive Mx1 promoter. Similar to pharmacological inhibition by

zIETD, isolated and cultured mouse EPC from spleen lacking caspase-8 had reduced abilities to form fibronectin-adherent cells compared with wildtype cells *in vitro*. Because EPCs come from the bone marrow and float in the circulatory system, mouse EPCs can be easily isolated from the bone marrow. EPC are separated by ficoll density centrifugation. Blood flow recovery and capillary density in the hind limbs after ischemia were reduced in samples with intravenously injected caspase-8-deficient BMC cells (Fig. 3.7.4). This requirement of caspase-8 for progenitor cell-mediated postnatal neovascularization extends previous findings demonstrating an essential role of this specific caspase for the development of the mouse embryonic yolk sac vasculature and circulation (Kang, Ben-Moshe et al. 2004). Additionally to integrin α5 downregulation in caspase-8 deficient BMCs, integrin α4 null mice have defects in placental and cardiac development resulting as well in embryonic lethality (Yang, Rayburn et al. 1995) and may contribute to defective embryonic vessel formation in caspase-8-deficient mice. Accordingly to our previous results using pharmacological inhibitors for Caspase-8, homing proteins like integrin alpha5, alpha4 and CXCR4 receptor were significantly reduced in conditional caspase-8 knockout BMCs (Fig.3.7.6a,b,d). These results support once again a novel apoptosis-unrelated role of caspase-8 for endothelial precursor cells in a cell-autonomous fashion. Our *in vivo* data using caspase-8 deficient cells underline the adhesion and migration disturbance as well as the limited homing and neovascularization capacity of cultured zIETD-treated human EPC. Thus, this study discloses an apoptosis-unrelated mechanism by which caspase-8 signaling interferes with angiogenic cell processes mediated by progenitor cell administration.

4.8 Cbl-b is a novel target of caspase-8

The question remained how caspase-8 regulates the fibronectin receptor integrin α5β1 and CXCR4. We tried to further identify the mechanisms, by which caspase-8 activity contributes to preservation of the fibronectin α5β1 and CXCR4 receptor surface expression and interferes with integrin and cytokine signaling. It is conceivable that caspase-8 continuously degrades inhibitors of integrins and

chemokine signaling such as ubiquitin-ligases like Cbl-b or regulators of the family of small GTPases. Three mammalian homologue proteins are characterized of the Cbl-family: c-Cbl, Cbl-b and Cbl-c. They are ubiquitin ligases and act as negative regulators of signaling responses with receptor tyrosine kinases, phosphatases, other ubiquitin ligases and adaptor proteins to influence cell proliferation, survival and migration. Cbl recruit ubiquitin-carrier enzymes or ubiquitin-conjugating enzymes as well as direct the multi-ubiquitination of various proteins and promote their sorting for lysosomal degradation (reviewed in (Schmidt and Dikic 2005)). Based on the publication of Cbl-mediated ubiquitination of the integrin α5 subunit in calvarial osteoblasts (Kaabeche, Guenou et al. 2005), we analyzed the expression of the major Cbl proteins involved in T-cell receptor signaling (Thien and Langdon 2005). Interestingly, c-Cbl is highly expressed in $CD34^+$ cells, contrary to high levels of Cbl-b in EPC. However, both ubiqutin ligases are expressed at low level in HUVEC and monocytes. This cell or tissue distributions may reflect the different phenotypes in mice depleted of c-Cbl and Cbl-b (Naramura, Jang et al. 2002). Interestingly, also bone marrow-derived IgE-activated mast cells express high levels of Cbl-b while deficiency leads to cell growth and retardation in receptor internalization (Gustin, Thien et al. 2006). Indeed, the present study provides experimental evidence that Cbl-b is a target of caspase-8. Several in vitro experiments indicate that caspase-8 cleaves the molecular adaptor protein Cbl-b using different truncated constructs (Fig.3.9.7). Inhibition by zIETD leads to downregulation of integrin α5 protein and β1 cell surface reduction in EPC. In contrast, pharmacological zIETD treatment results in Cbl-b upregulation (Fig.3.9.2). These data are consistent with the reduction in α5 integrin expression after caspase-8 inhibition shown by western blot and fluorescence images. The fibronectin receptor subunit integrin α5 was previously shown to be regulated by post-transcriptional modification coupled to proteasome degradation directly by Cbl-b in osteoblasts (Kaabeche, Guenou et al. 2005). We assumed that α5 is a multiubiquitiylated target protein for degradation (Ciechanover 2005) whereas integrin β1 is only monoubiquitiylated to recruit other signaling proteins (Hicke 2001). Therefore, provided by experimental evidence that caspase-8 cleaves the molecular adaptor protein Cbl-b, we assume that the interaction between

Cbl-b and integrin α5 might contribute to the inhibitory effects shown in *in vitro* adhesion and migration as well as *in vivo* neovascularization capacity of caspase-8-deficient bone marrow cells and leading to interference with intracellular signaling by blocking functional integrin signaling.

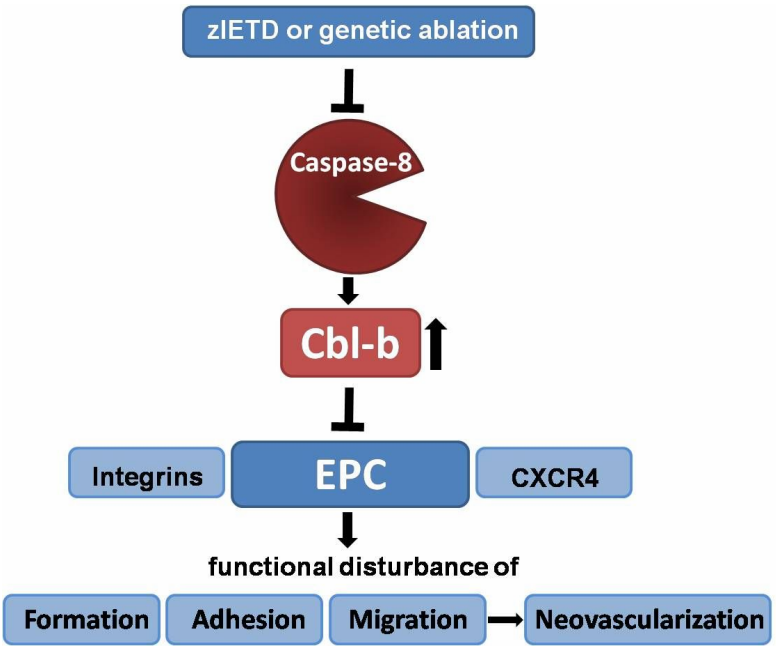


Figure 4.8.1: Caspase-8 is involved in proper EPC function

This schematic illustration reflects our results and opinion about caspase-8 function in EPC. Even we can not exclude the involvement of other signalling proteins so far, we demonstrated that Cbl-b is a target of caspase-8. Adhesion and migration proteins like integrins and CXCR4 are regulated in this signalling process dependent on the protein levels of caspase-8 and Cbl-b. Neovascularization is inhibited after caspase-8 inhibition/gene deletion and is increased after Cbl-b gene deletion.

4.9 Cbl-b inhibition promotes angiogenesis in vitro and in vivo

To demonstrate the role of Cbl-b in angiogenesis, we used Cbl-b-deficient BMCs for in vivo assays. Interestingly, these BMCs showed higher levels in integrin α5, CXCR4 and VEGF-R2 (KDR) (data not shown). Furthermore, Cbl family members function as

adaptor proteins downstream of CXCR4 by forming multiprotein signaling complexes in response to SDF-1α in outside-in signaling (Chernock, Cherla et al. 2001). Our findings in receptor surface reduction after caspase-8 inhibition in EPC and caspase-8-deficient bone marrow cells support our opinion about a regulating link between caspase-8 and Cbl-b which was observed in increased angiogenesis in matrigel and neovascularization assays by Cbl-b-deficient bone marrow cells. Controversially, recent publication demonstrates that Cbl-b-deficient bone marrow-derived mast cells produce significant higher levels of inflammatory cytokines TNF-α, IL-6 and MCP-1 compared to wildtype and c-Cbl$^{-/-}$ cells (Gustin, Thien et al. 2006). Probably, bone marrow derived cells from Cbl-b$^{-/-}$ mice act in a similar way by stimulating and positively supporting vessel growth with enhanced adhesion and increased chemokine receptors expression. Furthermore, the negative regulation of angiogenesis by Cbl-b was confirmed by siRNA downregulation in endothelial cells resulting in functional disturbance of sprouting activity and migration capacity (Fig.3.10.2 and 3.10.3). Cbl-b inhibition by siRNA significantly increased sprout length and migration activity in endothelial cells demonstrating a similar importance like in EPC. As recently published caspase-8 interacts with Src tyrosine kinase to promote adhesion (Finlay and Vuori 2007). Interestingly, Src and Cbl-b are important for activation of integrins and actin-associated proteins in osteoclast function and differentiation (Horne, Sanjay et al. 2005) supporting our results of positive caspase-8 downstream signaling in EPC. Additionally, Cbl-b promotes the ubiquitination of phosphatidylinositol 3-kinase (PI3K) (Fang, Wang et al. 2001). Inhibition of PI3K reduces chemokine-induced migration and adhesion of progenitor cells via β2-integrins and impedes neovascularization of ischemic tissue (Chavakis, Carmona et al. 2008). Furthermore, it was shown that c-Cbl ubiquitin ligase negatively regulates the small GTPase Rap1 activation (Shao, Elly et al. 2003) important for cell adhesion via integrin regulation (Bos 2005) and cell-cell function formation (Kooistra, Dube et al. 2007). However, despite the obvious relevance of a proper regulation of adhesion molecules for the retention of circulating precursor cells to target tissues, a role of Cbl-b for neovascularization-promoting progenitor cell function is not known.

Besides Cbl-b, caspase-8 could promote adhesion, migration and neovascularization in EPC in vivo through other putative targets. Additionally, Caspase-8 promotes calpain activity which is implicated in adhesion, lamellipodial movement cycles and activation of rac important for cytoskeleton remodeling (Franco and Huttenlocher 2005). High expression of Calpain was detected in our microarray data in EPC, HUVEC and monocytes but not in hematopoietic $CD34^+$ cells (data not shown). Recently, Senft et al. found that phosphorylated caspase-8 interacts with the p85 subunit of Phosphatidylinositol 3-kinase (Senft, Helfer et al. 2007). Interestingly, as mentioned before, Cbl-b can also bind the p85 subunit of PI3K (Fang, Wang et al. 2001). Caspase-8 and Cbl-b may possibly compete with each other under specific conditions. Furthermore, other possible targets affecting integrin function include kindlins, which are indispensable for cytoskeleton organization and function, cell-matrix adhesion (Larjava, Plow et al. 2008) and bidirectional signaling via integrins (Montanez, Ussar et al. 2008) and can inhibit integrin-dependent adhesion and migration. But possible regulation by non-apoptotic caspase-8 activation has to be investigated so far.

4.10 Non-apoptotic caspase-8 regulation in EPC

After demonstrating a novel non-apoptotic role of Caspase-8 in EPC incorporation, we questioned what might regulate caspase-8 expression in an apoptosis-independent way. The limited caspase-8 activation may occur independent of pro-apoptotic TNF or Fas receptor stimulation because no apoptosis was induced in the cells. As previously shown, caspase-8 can interact with the long isoform of cellular Flice-like inhibitory protein ($cFlip_L$) (reviewed in (Budd, Yeh et al. 2006)), which allows for selective degradation of substrates within a close local subcompartiment (Lamkanfi, Festjens et al. 2007). This means in close distance to the cell membrane where different complexes are built up besides the DISC (Death-inducing signalling complex). Recently, Sun et al. identified a novel antiapoptotic protein complex containing glycogen synthase kinase-3, DDX3 and cellular inhibitory protein-1 which inhibits apoptotic caspase-8 activation by impeding DISC formation. The detailed

protein complex coordination is still unknown (Sun, Song et al. 2008) but could be an explanation for upstream non-apoptotic caspase-8 regulation in EPC. Understanding the signals involved in the regulation of low grade caspase-8 activity and caspase-8 mRNA processing may identify upstream mechanisms of the caspase-8-dependent vasculogenic progenitor cell function. Additionally, besides intracellular signaling by protein complex formation, attraction of EPC to ischemic tissue could be another caspase-8 regulating process. TGF-β1, TNF-α, IL-1, IL-6 and IL-8 were localized in myocardial ischemic tissue and involved in inflammatory processes (reviewed in (Sharma and Das 1997)). Interestingly, exposure to the pro-inflammatory cytokine IL-6 increased vascular expression of α5 and β4 integrin subunits of astrocytes in the central nervous system (Milner and Campbell 2006). Additionally, interleukin-6 act as an angiogenic factor and mediates proliferation and migration of endothelial cells (Yao, Zhai et al. 2006). Furthermore, IL-6 enhances migration, development and repopulation of human stem cells by increasing CXCR4 surface expression (Lapidot 2001). Recent publication suggests that blood-derived EPC are recruited by IL-6 caused by ischemic or inflammatory areas (Fan, Ye et al. 2008). In contrast to TNF-α (data not shown), IL-6 increased the number of fibronectin-adherent endothelial precursor cells from human peripheral blood. Based on these results, we analyzed protein expression of Cbl-b and integrin α5 subunit surface expression. We found decreased Cbl-b protein levels after increasing IL-6 concentration. Interestingly, integrin alpha5 surface expression was increased with IL-6 stimulation. These results indicate that cytokine stimulation could positively influence EPC recruitment showing in Cbl-b and integrin α5 change. However, causal relevance of caspase-8 in this process could not be proved by a rescue experiment.

5. Conclusion

Vasculogenesis as well as angiogenesis are important for postnatal development of blood vessels. Peripheral blood or bone marrow-derived endothelial precursor cells are used in clinical trials for therapeutic enhancement of postnatal neovascularization in patients suffering from coronary artery diseases. The vasculogenic potential of the precursor cell population depends on the appropriate retention of the infused cells to the ischemic tissue. However, cell-autonomous mechanisms regulating the attraction and retention of circulating cells in inflammatory tissue are not well understood. Caspases belong to a family of pro-apoptotic enzymes. Beyond cell death signals, caspase proteases additionally regulate non-apoptotic processes like cell morphology and migration in many cell types.

The isoform Caspase-8 is essential for embryonal vasculogenesis in conditional knockout mice. In this study, we identified a novel apoptosis-unrelated role of Caspase-8 in circulating and bone marrow-derived cells for vascular repair. Caspase-8-specific inhibition abrogated the *ex vivo* formation of EPC from human peripheral blood. Moreover, Caspase-8 inhibition disables EPC migration and adhesion to different matrices and decreases the cell surface expression of the fibronectin receptor subunit integrin α5 and the chemokine receptor CXCR4. *In vitro* and *in vivo* studies using bone marrow mononuclear cells derived from inducible Caspase-8-deficient mice revealed an essential role of Caspase-8 for EPC formation and neovascularization enhancing capacities of progenitor cells. Caspase-8 activity appears to be required for maintaining responses to matrix interaction and chemoattractants of EPC.

Additional studies showed that the E3 ubiquitin ligase Cbl-b, a negative regulator of cell adhesion molecules including integrin α5, is present in EPC at low protein levels under basal conditions, but markedly increases upon Caspase-8 inhibition. *In vitro* assays and overexpression studies in intact cells confirmed Caspase-8-dependent degradation of Cbl-b, providing a potential requirement for Caspase-8-regulated adhesion. Indeed, neovascularization of matrigel plugs was enhanced in mice lacking Cbl-b. Moreover, Cbl-b degradation in the presence of active Caspase-8 prevents the

down-regulation of integrin α5 and is associated with an enhanced vasculogenic activity of progenitor cells in hind limb ischemia. The identified upstream regulation of caspase-8 by cytokine IL-6 is only one possibility for fine-tuning the non-apoptotic enzymatic activity.

In summary, this study shows a novel essential role of Caspase-8 for proper EPC adhesion-related signaling. Caspase-8 is involved in the function of adhesion molecules by regulation the E3 ubiquitin ligase Cbl-b. Strategies to improve survival of therapeutic injected progenitor cells by using caspase inhibitors should be addressed with caution. Because of the broad spectrum of activity of caspase-8, downstream targets of this caspase isoform and Cbl-b should be in more focus for therapeutic pretreatment to improve neovascularization of myocardial and ischemic tissue.

6. Abbreviations

Ac-LDL	acetylated low-density lipoprotein
Bp	Base pairs
BSA	Bovine serum albumin
CAD	Coronary artery disease
cDNA	complementary DNA
Ct	C-terminus
DAPI	4`,6-diamidino-2-phenylindole
DISC	Death-inducing signaling complex
DNA	Desoxyribonucleic acid
DTT	Dithiothreitol
e.g.	for example
EC	Endothelial cells
ECM	Extracellular matrix
EDTA	Ethylendiamintetra acetic acid
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial precursor (progenitor) cell
et al.	and others
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
Fig.	Figure
FITC	Fluorescein isothiocyanate
Flip	Flice-like inhibitory protein
g	grams or g force
GAPDH	Glycerinaldehyd-3-Phosphat-Dehydrogenase

h	hour(s)
HEK293	Human embryonal kidney epithelial cells
HMVEC	Human microvascular endothelial cells
HUVEC	Human umbilical vein endothelial cells
i.v.	intra venous
kDa	Kilodalton
KDR	VEGF receptor 2
min	minutes
MNC	Mononuclear cells
mRNA	Messenger ribonucleic acid
n	Number of experiements
n.s.	Not significant
Nt	N-terminus
OD	Optical density
PBS	Phosphate buffered saline
PIGF	Placenta growth factor
PMSF	Phenylmethylsulfonylfluorid
RT	Room temperature or reverse transcription
SDS	Sodium-dodecyl-sulfate
SEM	Standard error of the mean
siRNA	Small interfering RNA
TNFα	Tumor necrotic factor alpha
VEGF	Vascular endothelial growth factor
VEGF	Vascular endothelial growth factor
vWF	Von-Willebrand factor
Wt	wildtype

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Lebenslauf

Dörte Scharner

Calvinplatz 1
63263 Neu-Isenburg
Tel: 06102 / 294026
Email: Doertescharner@web.de

geboren am 01.01.1980 in Stralsund

ledig

Schul-und Hochschulausbildung

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1986 - 1998	Grundschule und Gymnasium Barth mit Abschluss der **Allgemeinen Hochschulreife** Note: 2.9

Vorträge und Posterpräsentationen

November 2007	Teilnahme mit Präsentation am American Heart Association Kongress - Orlando
	"Caspase-8-dependent cleavage of the E3 ubiquitin ligase Cbl-b regulates integrin expression and adhesion capacity in endothelial progenitor cells"
April 2007	Teilnahme mit Präsentation an der 73. Jahrestagung- Deutsche Gesellschaft der Kardiologie- Mannheim
	„Caspase-8 degradiert die E3 Ubiquitin-Ligase Cbl-b und reguliert dadurch die Integrinexpression und Adhäsion endothelialer Progenitorzellen"
November 2006	Teilnahme mit Präsentation am American Heart Association Kongress - Chicago
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Publikationen

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ATP- independent contractile proteins from plants.

Nature Material; 2003

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Frankfurt am Main, den 09.03.2009 _____

Dörte Scharner